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STUDIES ON PROTEIN DENATURATION BY SURFACE CHEMICAL METHOD

I. THE RELATIONSHIPS BETWEEN MONOLAYER PROPERTIES AND UREA DENATURATION OF LYSOZYME

By KOZO HAMAGUCHI

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(Received for publication, May 2, 1955)

In the series of our papers on the synthetic protein analogues, we have mainly concerned with the monolayer properties of synthetic polypeptides as the model of natural proteins (*1–7*). It remains to be solved that to which extent the knowledge obtained with synthetic polypeptides can be applied to the elucidation of the behaviour of protein monolayers and that what are the differences between both. Comparing with proteins, synthetic polypeptides are simple linear high polymers and have no such a highly organized structure as proteins ; they resemble rather the denatured proteins in structure. The fact that must be first noticed here is that whether the monolayer properties would be reflected by the specificity or organization in protein structure or not. There have been so far many investigations upon the protein monolayers at air-liquid or liquid-liquid interfaces (*8*). It was, however, tacitly assumed that all the proteins are spread with the configuration of β-keratin type at the interfaces, and the individuality of each protein film, that is, the relation between the monolayer properties and their composition of amino-acid, was scarcely examined.

To clarify the foregoing subject, the problem of whether or not the denaturation of proteins in spreading solution is accompanied by the change in their monolayer properties must be first examined. However, little work has been reported on such an investigation. Schaefer (*9*) found that when the spreading solution of protein is treated with heat or ultraviolet rays, their expansion patterns were different from the untreated protein. Ställberg (*10*) reported that the area of the film of serum albumin was 15 per cent greater than the native protein, if the spreading solution was heated prior to spreading. Dervichian (*11*) also examined along the same line. On the other hand, Bull and

Neurath (*12*) found that denatured proteins cannot be spread as readily as native ones since the inter- or intramolecular cohesion of their groups is so strong that it is not overcome by the forces which causes the spreading of the film. Recently, Kaplan and Fraser (*13*, *14*) examined the " Devaux fibre " microscopically which was formed by compressing up the protein monolayer and found that its structure was quite different by whether or not the spreading solution was treated with heat, ultraviolet rays or urea prior to spreading.

The reasons why lysozyme was especially chosen as a specimen among the other proteins were as follows: (*1*) The amino-acid composition of this protein has been definitely determined (*15*, *16*). (2) This protein has a very rigid structure and is difficult to be denatured because of the five intrapeptide disulfide bridges existing in rather small molecule of the molecular weight of 14,800 (*17*). So it is considered that lysozyme is a very convenient one to examine the degree of denaturation.

In this paper the monolayer properties of lysozyme on water surface, especially its relation to the urea denaturation are described.

EXPERIMENTAL

Materials and Methods

The crystalline lysozyme was kindly provided by Prof. S. Akabori and Mr. K. Ohno, who prepared it by direct crystallization method and recrystallized for four times.

Surface pressure was measured by Sasaki's modification (*18*) of Wilhelmy hanging plate method. The stable monolayers of lysozyme were formed on the substrate at its isoelectric point (about 10.5), of which pH was adjusted by 0.01 moles of potassium carbonate. The spreading solution of native lysozyme was prepared by dissolving it in *N*/1000 hydrochloric acid solution which was added by 2 per cent of isopropyl alcohol in order to assist the spreading. As the urea solution of lysozyme could not be spread, 40 per cent isopropyl alcohol was added prior to spreading after the protein was completely dissolved in urea solution of a given concentration. The urea used was a commercial product of Toyo Koatsu Kogyo Co. It was used after purification by repeated recrystallization and adsorption by active carbon. In the case of heat denaturation, 2 per cent of isopropyl alcohol was added after the protein solution in *N*/1000 hydrochloric acid was heated in a water bath regulated to desired temperatures and then used.

RESULTS

Fig. 1 shows the surface pressure-area (F-A) curves obtained for the monolayers which were spread from the solutions of lysozyme which contained urea in different amounts. All the curves in this figure were obtained for the films which were kept on the substrate of pH 10.5 for 30 minutes before measurements. It was found from this figure that the F-A curve of the film obtained from the urea solution of 5 moles per litre was not so different from that obtained from *N*/1000 hydrochloric acid solution. When the urea content in lysozyme solution was increased to 8 or 10 moles per litre, the F-A curves were shifted to much larger area and began to develope from the area of about 15 Å^2/ residue.

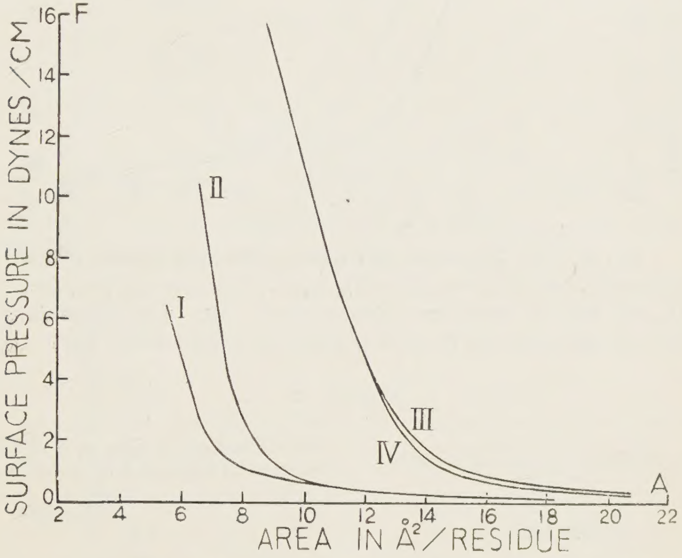

Fig. 1. The F-A curves of lysozyme films spread from *N*/1000 hydrochloric acid solution (Curve I), from 5 moles urea solution (Curve II), from 8 moles urea solution (Curve III), and from 10 moles urea solution (Curve IV). The elapsed time between spreading and the start of compression is 30 minutes. (23°)

The conditions for the curves shown in Fig. 2 were all the same as those for Fig. 1 except that the films were kept on the surface for twenty hours. By comparing the F-A curves in Fig. 1 with those in Fig. 2, we found that the F-A curves of the films aged for twenty hours were remarkably expanded in the case of *N*/1000 hydrochloric acid and 5 moles urea solution, even though the expansion of the film from the latter was greater than that from the former. However, when the films were spread from 8 or 10 moles urea solutions the aging effect was not so marked.

The aging effect may be indicated by the differences in the area at 2 dynes/cm. after 30 minutes aging and 20 hours aging which are shown in Table I.

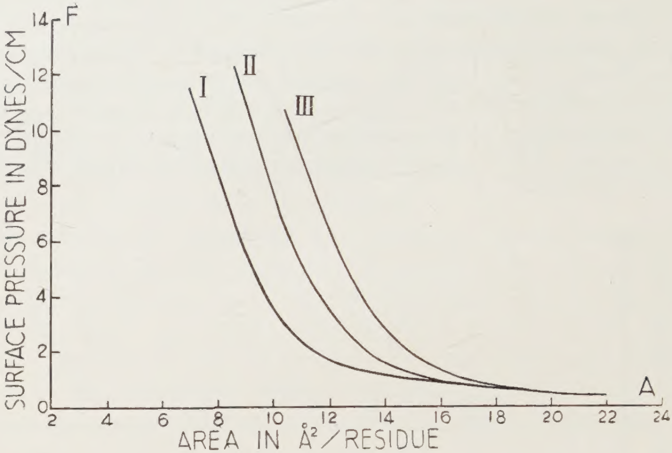

FIG. 2. The F-A curves of lysozyme films spread from, $N/1000$ hydrochloric acid solution (Curve I), from 5 moles urea solution (Curve II), and from 10 moles urea solution (Curve III). The elapsed time between spreading and the start of compression is 20 hours. (23°)

TABLE I

The solvents of spreading solution	The difference in area at 2 dynes/cm. between 20 hours and 30 minutes aging.
	($Å^2$/*residue*)
$N/1000$ HCl	4.4
5 M urea	4.9
10 M urea	1.5

From Fig. 1 the relation of the areas at constant pressure against the urea concentrations in the spreading solutions may be obtained and is shown in Fig. 3. It was found that the sudden increase in area occurred at the concentration of about 6 moles per litre urea. Fig. 4 shows the effect of heat denaturation on the F-A curves; Curve I in this figure was that for the film obtained from the $N/1000$ solution of hydrochloric acid and Curve II for that from the solution which was heated in the water bath of 65°±3°. for two hours prior to spreading. There was no marked difference between them.

DISCUSSION

The behaviour of the monolayers of lysozyme is determined by

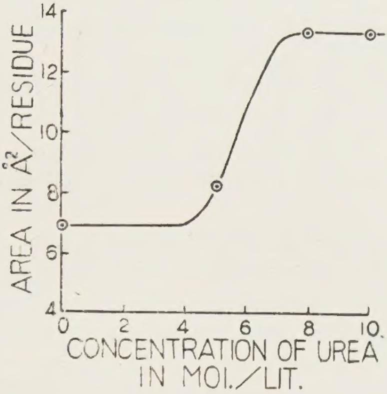

FIG. 3. The relationship between the areas at 2 dynes/cm. and the urea concentrations in the spreading solution.

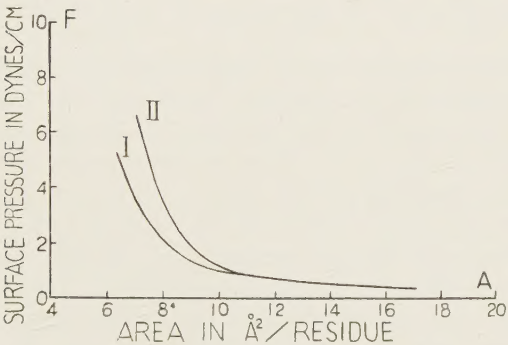

FIG. 4. The effect of heat denaturation on F-A curves of lysozyme. Curve I, spread from *N*/1000 hydrochloric acid solution ; Curve II, spread from *N*/1000 hydrochloric acid solution which is heated in the water bath of 65°±3° for two hours beforehand.

several factors as follows:

(1) *The Spreading Solution*—(a) The denaturing agents such as urea, heat, as well as the pH's of the solution affect the monolayer properties; (b) The aging of the spreading solution also affects the behaviour of its monolayer. For instance, after the solution of lysozyme in *N*/1000 hydrochloric acid was preserved in a kelvinator at about

0° for 3 weeks, the F-A curve obtained from it developes from larger area than the fresh solution. The lysozyme in alkaline solution is more unstable than that in the acid solution.

(2) *The Substrate*—(a) The pH of the substrate; (b) The temperature of the substrate. The F-A curve of lysozyme is affected markedly by the temperature of the substrate and is shifted to larger area as the temperature rises, details of which will be reported in the following paper.

(3) *The Time Elapsed to the Measurements after the Spreading Affects the Monolayer Properties*—As described above, most authors assumed that the proteins are spread at interfaces with the configuration of β-keratin type and that the protein molecules suffers the most drastic alternation on their configuration when spread at interfaces. On the other hand, there have been some evidences that enzymes continue to display their activity even if they are spread at interfaces (*19*, *20*). Therefore, these facts suggest that some regularities existing in native molecules remain unchanged even in the spread monolayers. These two apparently opposing resuls seem to be very interesting for the investigations of protein monolayers.

It is generally accepted that the highly organized structure of protein molecules is maintained by disulfide bonds and the other secondary bonds such as hydrogen bonds, hydrophobic bonds, salt linkages, or London dispersion forces (*21*). Now, we assume that only the disulfide bond is the most important factor which is involved with the easiness of denaturation and with the form of the molecules after denaturation. The total number of amino acid residues (N) and the number of disulfide bonds (n_s) of each protein are summarized in Table II (*22*).

The ratio $N/(2n_s+1)$, which gives the average number of amino-acid residues separated by every disulfide bond, may be assumed as a measure of the easiness for danaturation. Then, the smaller value of $N/(2n_s+1)$ have proteins, the more they are difficult to be denatured and the more compact are their structures. In Table II proteins are arranged in the order that value of its ratio increases from top to bottom. Of course, in order that the above treatment would be possible, the dissulfide bonds are equally distributed in the polypeptide chain and are of intrapeptide linkages. Although so simple are these considerations, some experimental facts described below suggest that the value of $N/(2n_s+1)$ may be considered to give a measure of easiness for protein denaturation.

(1) Insulin, which according to Rothen (*24*), does not lose its

TABLE II

Proteins	The number of S-S bonds (n_s)	The total number of amino-acid residues (N)	$N/(2n_s+1)$
1. Insulin (bovine)	6	103	8
2. Lysozyme	5	123	11
3. Ribonuclease	4	122	14
4. Serum albumin (human, bovine)	16	602	18
5. β-Globulin	13	722	27
6. Chymotrypsinogen	3	206	29
7. β-Lactoglobulin	4	365	41
8. γ-Globulin	15	1305	42
9. Taka-amylase (*23*)	4	457	51
10. α-Globulin	13	1519	56
11. Pepsin	2	306	69
12. Edestin	1.5	396	99
13. Ovalbumin	1	387	129
14. Myoglobin (horse)	0	143	143
15. Hemoglobin	1.3	541	150
16. Gelatin	0	—	—
17. Collagen	0	—	—

hormone activity even in the state of spreading monolayer.

Cumper and Alexander (*25, 26*) obtained the area of 7.9 Å^2/ residue for the insulin monolayer by surface viscosity measurements. This value is far smaller than the area of about 15 Å^2/ residue usually obtained for protein monolayers such as ovalbumin. Dien and Bull (*27*) found that the F-A curves are changed with time elapsed from spreading to measurements. These facts suggest that insulin is difficult to be denatured by surface.

(2) Lysozyme which is very difficult to be denatured as shown in the present experiment.

The monolayer of lysozyme was already examined by Bull (*28*), who pointed out that this protein did not form surface films.

(3) Ribonuclease, of which denaturation is, according to Anfinsen (*29*), not due to the chain unfolding but due to the aggregation of the molecules.

This protein also resists against heat.

(4) The proteins which are described below serum albumin in the table are the usual ones of which monolayer properties have been so far investigated and give the films of the same type. However, the differences between e.g., serum albumin and ovalbumin are clearly shown by Tachibana and Inokuchi (*30*), who found that the area from which viscoelastic properties appear in the latter film was much larger than that in the former film and concluded that the film molecules of serum albumin have more compact form than those of ovalbumin. This fact can be well understood from the table. Another different point between serum albumin and ovalbumin is that whereas the denaturation of the former protein is reversible, this is not the case in the latter.

From the above consideration it is expected that lysozyme is quite resistant to the denaturing action.

For the F-A curves obtained with the films of lysozyme spread from its solution in *N*/1000 hydrochloric acid (Fig. 1, Curve I), The area from which the surface pressures increase suddenly is much smaller than the area obtained with the usual protein films, such as ovalbumin, *i.e.*, about 15 Å^2/ residue (*8*), and corresponds to the area of insulin film (7.9 Å^2/ residue), which was found by Cumper and Alexander (*25*, *26*) from the surface viscosity measurements. When the films are spread from *N*/1000 hydrochloric acid solution and are stood for 20 hours before measurements, the F-A curve (Fig. 2, Curve I) expands but yet the area is smaller than 15 Å^2/residue. These facts suggest that the film of lysozyme is made of the slightly denatured molecules when native protein is spread on the surface and may not be completely denatured by surface even if the film is aged for 20 hours.

In the case of the lysozyme film spread from 5 moles urea solution, the F-A curve (Fig. 1, Curve II) is not so different in area from that obtained with *N*/1000 hydrochloric acid solution. However, when the film spread from 5 moles urea solution are stood on the surface for 20 hours, the film (Fig. 2, Curve II) expands and its limiting area tends to the area of 15 Å^2/residue and the aging effect is the most marked (Table I). These facts suggest that the lysozyme molecules which are slightly denatured by urea suffers greatly the following surface denaturation.

The films obtained from 8 or 10 moles urea solutions do not expand markedly even if the film is aged for 20 hours before measurements (Table I) and the F-A curves develope from the area of bout 15 Å^2/residue. This is because of the fact that the lysozyme molecules in these urea solutions are completely denatured.

The curve shown in Fig. 3 shows the degree of denaturation to which extent the lysozyme molecules in each urea solution are denatured. The curve resembles that obtained by Neurath, Cooper and Erickson (*31*) using certain solubility tests as the measure of activity of the urea and that by Klotz, Triwusch and Walker (*32*) who obtained the desorption curve of methyl orange adsorbed on the protein by urea. From this figure it is found that the denaturation does not take place in the urea solutions of the concentration range from zero to five moles and that the denaturation proceeds suddenly at the concentration of about 6 moles and then complete at the concentrations of more than 8 moles urea. These facts lead to the important conclusion that in the case of the proteins with very rigid structure, such as lysozyme, they cannot be spread as completely unfolded films unless they are completely or nearly completely denatured in the solution beforehand.

Whether lysozyme in *N*/1000 hydrochloric acid solution is heated previously or not, do not affect the F-A curves as shown in Fig. 4. This result shows that lysozyme in acid solution is very stable to heat. Preliminary experiments showed that it is unstable in alkaline solution, details of which will be described in the next paper.

Bull (*8*), as described above, pointed out that the area at minimum compressibility obtained from the relations of compressibility and area, becomes 0.8 m^2./mg. (15.3 Å^2/residue), irrespective of the species of proteins and that this value corresponds to the area of the closest packed polypeptide chain, which has been also ascertained for synthetic polypeptides (*1, 2, 4*). However, such a case may only be realized with proteins which are readily denatured by surface and not with proteins of somewhat rigid structure such as insulin, lysozyme, or ribonuclease, as expected from Table II. Even for the proteins wihch are readily denatured, such as ovalbumin, the denaturation in their spreading solution affects the monolayer properties as described above (*9–14*). These facts suggest that some structural regularities in native protein molecules remain in the film without destruction by surface forces.

In the case of cellulose acetate, there are evidences that the spreading solutions play an important role for the determination of the monolayer properties. Abribat and Pouradier (*33*), for instance, found that the F-A curves of cellulose acetate films were changed by whether they were spread from its acetone solution or from the solution in the mixture of methylene chloride and alcohol, and explained that the configuration which are assumed by the film molecules depends on the spreading solution. The similar results were also found by Borgin and

Johnson (*34*) for the monolayers of cellulose triacetate spread from the solution in chloroform-alcohol mixture.

It is found from the present experiment that monolayer technique is useful for the examination of the degree of denaturation in protein solutions.

SUMMARY

The monolayer properties of lysozyme in relation to its urea denaturation were examined. The results obtained were as follows:

1. The F-A curves of lysozyme film spread from $N/1000$ hydrochloric acid solution had much smaller limiting area compared with the proteins, such as ovalbumin. However, when lysozyme was spread from 8 or 10 moles urea solutions, the area expanded to that of about 15 Å^2/residue. From the relations between areas at constant surface pressure and the urea concentrations in the spreading solutions we could estimate the degree of urea denaturation in the solutions.

2. It was shown by the monolayer technique that lysozyme in acid solution resists against heat.

3. The anomalies in the monolayer properties of lysozyme was discussed in terms of its high content of disulfide bond.

The author expresses his hearty thanks to Prof. T. Isemura for his kind guidance throughout the present work, and to Prof. S. Akabori and Mr. K. Ohno in Faculty of Science, Osaka University who kindly supplied the valuable sample to him.

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ON THE CYANIDE-COMPOUNDS OF NATIVE HEMOGLOBIN AND MYOGLOBIN

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(Received for publication, May 7, 1955)

A good deal of observation has already been done on the cyanide-compounds of ferrous hemoglobin and myoglobin. Anson and Mirsky (*1*) observed two absorption bands at 561 and 535 mμ when KCN was added to a solution of ferrous hemoglobin at pH 6–10. These absorptions, however, disappeared within a very short time. Balthazar (*2*) also reported the temporary appearance of these two absorption maxima when CN-compound of ferric hemoglobin was reduced by $Na_2S_2O_4$ in an alkaline solution. Similar observations were reported by Kiese (*3*) using myoglobin. Based on the fact that CN-compound of ferrous hemoglobin is diamagnetic, Stitt and Coryell (*4*) suggested that the nature of the bonding between CN and ferrous hemoglobin would be covalent.

However, in view of the short life of the two absorption bands mentioned above, some doubts may be entertained as to whether or not CN actually combines with native ferrous hemoglobin.

In connection to this problem, some interesting observations were made recently by the present author in the course of investigating the reaction of pseudomyoglobin formation in the reaction system of myoglobin-KCN-H_2O_2. The results of some typical experiments is presented in the following.

RESULTS AND DISCUSSION

Crystalline metmyoglobin prepared by the method devised in our laboratory (*5*) was used. When KCN was added to a metmyoglobin solution and subsequently $Na_2S_2O_4$ was added to the mixture, two distinct absorption bands appeared with their maxima at 565 and 535 mμ (Fig. 1, II) as already reported by Kiese. These two absorption maxima disappeared within about 2 minutes, and instead there appeared the ordinary absorption spectrum of reduced myoglobin (Fig. 1, III).

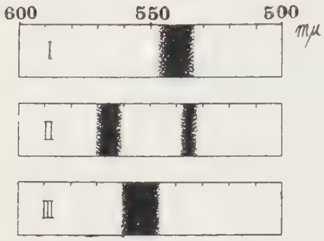

Fig. 1. Absorption spectra of myoglobin derivatives.

I. CN-compound of ferric myoglobin
II. CN-compound of ferrous myoglobin
III. Ferrous myoglobin

When, however, KCN was added to the solution of myoglobin in the reduced form, prepared from metmyoglobin by reduction with $Na_2S_2O_4$, no appreciable change occurred in the absorption figure. But, when small amounts of H_2O_2 was added to this reaction solution additionally, two absorption bands as shown by II in Fig. 1 appeared, persisting also for 2 or 3 minutes and changing thereafter into that of reduced myoglobin as before. This reversible change in the absorption spectrum caused by the addition of H_2O_2 could be repeated as long as $Na_2S_2O_4$ was present in sufficient amount in the reaction solution to reduce CN-compound of metmyoglobin into ferrous myoglobin.

These observations may be construed as indicating that CN actually combines with ferrous myoglobin. The fact that CN-compound of ferrous myoglobin can only transiently come into existence, and indeed only through CN-metmyoglobin by reduction, may be explained on the assumption that the affinity of CN to ferrous myoglobin is far weaker than that to ferric myoglobin. Thus, when CN-compound of ferric myoglobin was reduced, CN remained combined to myoglobin at that instant even in its ferrous state. When H_2O_2 is added to the solution of ferrous myoglobin in the presence of KCN, ferric myoglobin is formed and this ferric myoglobin is immediately converted into its CN-compound. In the next moment, this CN-compound of ferric myoglobin would be reduced to its ferrous state by $Na_2S_2O_4$ existing in the reaction solution and at this last stage of cyclic reaction, two absorption maxima at 565 and 535 mμ would transiently become observable.

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STUDIES ON POSTERIOR PITUITARY HORMONES

I. CHROMATOGRAPHIC SEPARATION OF OXYTOCIC AND PRESSOR ACTIVITIES ON ION EXCHANGE COLUMN*

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(Received for publication, May 7, 1955)

Several investigations have been made of the separation and identification of oxytocic and pressor activities of posterior pituitary gland, and recently, du Vigneaud *et al.* (*1*, *2* and *3*) prepared highly potent oxytocin and vasopressin preparations by the counter current distribution technics of Craig (*4*). For the preliminary purifications, however, they applied the method of Kamm *et al.* (*5*), which involved principally the use of fractional precipitation technics with organic solvents and had been extensively applied to the manufacturing of the hormone preparations for medical use. Potts and Gallagher (*6*) also announced a high degree of separation of the oxytocic from the pressor hormone by an adsorption process. By repeated adsorption with permutite, they were able to obtain solution in which the ratio of pressor activity to oxytocic activity was 20 : 1, but they described no preparation in which the pressor hormone was freed to the same extent from the oxytocic.

In the present work, not only partially purified posterior pitutitary hormone but also simple aqueous extract of the gland were chromatographed on columns of the carboxylic acid ion exchange resin, Amberlite IRC-50, and the practically complete separation of the two activities was achieved.

EXPERIMENTAL

Materials—Simple aqueous extract of the gland (*Preparation A*) was prepared as follows. 100 g. of U. S. P. posterior pituitary (purchased from the Armour Pharmaceutical Center, A Division of Armour and Company, Bradley, Illinoi.) was suspended in 10 liters of 0.25 per cent acetic acid. The mixture was gradually heated to a temperature of about 95° during half-hour period, cooled quickly and then filtered to remove the insoluble gland residue. Each ml. of the filtrate contained 9 units of oxytocic and equal number of units of pressor activity.

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* Presented before the Symposia on Biological Chemistry in Tokyo, October, 1953 : J. P. 16662, (1953).

The aqueous extract was further treated in the same way as described by Kamm *et al.* (*5*). The resulting white powder, which contained per mg. 13 units of both the two hormone activities, was stored in a refrigerator and subjected to a large scale chromatography as the partially purified posterior pituitary hormone (*Preparation B*).

Methods—Chromatography was carried out on columns of ion exchange resin, Amberlite IRC-50. The preparation of the resin and the operation of the columns were carried out according to the procedure of Hirs, Moore and Stein (*7*), and Tallen and Stein (*8*). Ammonium acetate buffer was used as the eluting agent, and effluent fractions were collected for analysis using a fraction collector described by Seki (*9*).

Protein measurements of effluent fractions were carried out with the modified Folin reagent (*13*). 2 ml. of the alkaline solution containing copper was added to 0.4 ml. of the effluent solution. After 10 minutes, 0.2 ml. of dilute Folin reagent was added to the mixture and allowed to stand for 30 minutes or longer. The intensity of the resulting colour was measured at 720 mμ by Photo Electric Colorimeter A. K. A. 5-D (Kotaki Seisakusho Co., Ltd.). Oxytocic activity was determined by the method of Coon (*10*), and pressor activity was measured on dogs anesthetized with sodium-phenobarbital.

Chromatography of Simple Aqueous Extract—Fifty ml. of preparation A (450 units) was cautiously placed on 1 × 14 cm. column, which had previously been equilibrated with 1/10 N ammonium acetate buffer (pH 4.5). It was eluted first with 2/10 N ammonium acetate buffer (pH 5.4) and then with 4/10 N ammonium acetate buffer pH 9.0). The rate of flow of the solvent through the column was 4–5 ml. per hour, and 5 ml. fractions of the effluent were collected for analysis. The results are shown in Fig. 1.

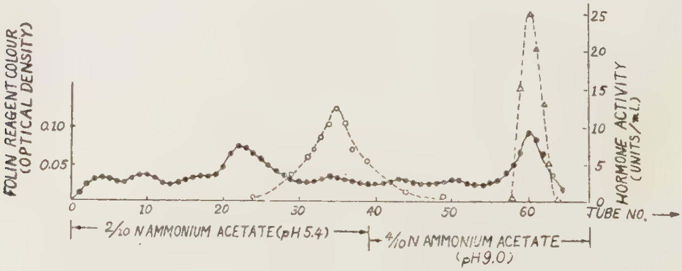

Fig. 1. Chromatography of posterior pituitary extract.
●—● Folin reagent colour. ○- - -○ Oxytocic activity.
△- - -△ Pressor activity.

Chromatography of the Partially Purified Hormone (*Large Scale Chromatography*)—A portion of 779.3 mg. of the *Preparation B* (10, 131 units) was dissolved in 200 ml. of 1/10 N

ammonium acetate buffer (pH 4.5), and placed on 3 × 35 cm. column equilibrated with the same buffer. The rate of flow was 50–80 ml. per hour and fractions of the effluent were 43.7 ml. in average. It was eluted first with 2/10 *N* ammonium acetate (pH 5.4) and then with 5/10 *N* ammonium acetate buffer (pH 9.0) as shown in Fig. 2.

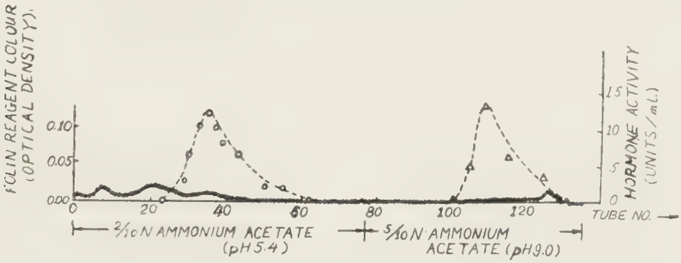

FIG. 2. Chromatography of the partially purified hormone.
●—● Folin reagent colour. ○- - -○ Oxytocic activity.
△- - -△ Pressor activity.

DISCUSSION

While du Vigneaud reported that the use of ion exchange resin could only afford unsatisfactory results for the purification of vasopressin (*3*), recent progress in the chromatographic analysis of amino acids and peptides suggested the applicability of the resin to the separation of the two posterior pituitary homones. Since it had been found that the isoelectic points of oxytocin and vasopressin were pH 7.7 and 10.9, respectively (*11*, *12*), preliminary experiments were made of the chromatographic behavior of the two hormones on the cation exchange resin, Amberlite IRC-50 (Fig. 3).

Thirty mg. of *Preparation B* was dissolved in adsorbing buffers as shown in Table I, and placed on 1 × 14 cm. column (10 ml. H-type resin) which had previously been equilibrated with the same buffer. The buffers with different concentrations and pHs were used as eluting agents and the effluent fractions were collected for analysis by the same procedure as described above (Fig. 3).

As shown in Fig. 3c, Na-type IRC-50 was not able to adsorb the hormones. When the 1/10 *N* ammonium acetate buffer (pH 6.0) was used as an adsorbing buffer, the pressor activity was adsorbed almost completely, whereas the oxytocic passed through the column without being adsorbed.

Therefore the chromatography as shown in Fig. 3d may be used as

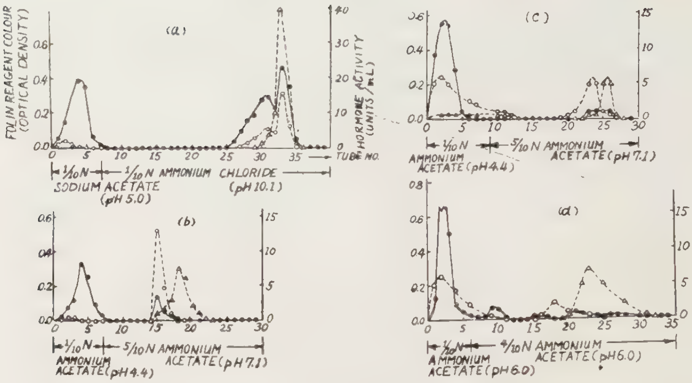

Fig. 3. Chromatography of the partially purified posterior pituitary hormones.

●—● Folin reagent colour. ○- - -○ Oxytocic activity.
△- - -△ Pressor activity.

TABLE I

Adsorbing buffers	Homone concentration (units/ml.)	Eluting buffers	
$N/10$ Sodium ecetate (pH 5.0)	14.5	$N/10$ Ammonium chloride (pH 10.0)	Fig. 3 (a)
$N/10$ Ammonium acetate (pH 4.4)	11.6	$N/10$ Ammonium acetate (pH 6.0)	Fig. 3 (b)
,, ,, (pH 4.4)*	11.6	$N/10$ sodium acetate (pH 7.1)	Fig. 3 (c)
,, ,, (pH 6.0)	82.6	$N/10$ Ammonium acetate (pH 6.0)	Fig. 3 (d)

* Only in this case, the resin was used as Na-type without preliminary equilibration with any buffer.

a simple procedure for the purification of vasopressin.

The pretreatment of the resin with 1/10 N ammonium acetate buffer of pH 4. 4 resulted in the practically complete adsorption of the two hormones together with relatively small quantities of the impurities. The chromatography as shown in Fig. 3a, therefore, can be applied to the purification of the hormone preparations.

Since the application of a single eluting buffer in chromatography as shown in Fig. 3 brought about no complete separation of the two hormone activities, the two sorts of ammonium acetate buffers with different concentrations and pHs were used successively as eluting buffers (Figs. 1 and 2), whereby satisfactory results were obtained not only for partially purified posterior pituitary hormone but also for simple aqueous extract of the gland.

SUMMARY

The chromatographic behaviors of the posterior pituitary hormones on the cation exchange resin Amberlite IRC-50 were investigated and the chromatographic separation of oxytocic and pressor activities was achieved not only for partially purified hormone but also for simple aqueous extract of the gland.

The authors wish to thank Dr. Masuichi Takino for his help in biological assays.

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BACTERIAL METABOLISM OF γ-AMINOBUTYRIC ACID

I. THE MODE OF ACTION OF DIETHYL DITHIOCARBAMATE ON THE FORMATION OF ADAPTIVE ENZYME*

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(Received for publication, May 16, 1955)

During the course of investigations on the physiological significance of the glutaminocarboxylase in a living body, a strain of bacteria has been isolated in our laboratory, which grew on the pure solution of γ-aminobutyric acid (γ-ABA) buffered at pH 7.0, and was determined as belonging to the genus of *Escherichia.* Indeed, it oxidizes as well as assimilates γ-ABA adaptively, since it may be utilized by the resting cells as C-and N-sources. It has been later ascertained that *Achromobacter sp.*, UH_3** as well as *Escherichia sp.*. is also able to assimilate γ-ABA adaptively and the former was mainly submitted to the present investigations.

Although it has been recently found by many workers that γ-ABA, is very widely distributed not only in animals, but also in plant kingdom very little is known on the metabolism of the acid, and no valid physiological function has been assigned to it. A possible approach to this problem appears to be the analysis of the mechanism of the oxidative assimilation of γ-ABA by means of resting cells of bacteria, which oxidizes it either adaptively or not. As described below, it has been clearly demonstrated that the oxidative assimilation of γ-ABA by these bacteria leads to the formation of a typical adaptive enzyme system, when they are incubated with the acid. Therefore it seems of interest to study the inhibition of enzyme formation in the presence of small amounts of streptomycin, 2, 4-dinitrophenol, or the like, which are capable of uncoupling the junction between the oxidation and assimilation of certain utilizable substances. Diethyl dithiocarbamate (DDC), known as a chelating agent, also seems to be one of the " uncouplers ", because the enzyme formation by bacterial oxidative assimilation of γ-ABA is evidently inhibited in the presence of DDC as well as streptomycin, 2, 4-dinitrophenol *etc.*, but it is reasonably conceivable that the inhibition by DDC could be attributed to a mechanism involving its chelation with some metal ions essential to the formation of adaptive enzyme system. A question which arises in attempts to determine the mechanism of the action of DDC, is whether some of heavy metal ions are necessary for the bacterial metabolism. As is described in the present communication, the results obtained on the addition of DDC, and of several heavy metal ions make it quite clear that the adaptive formation of the enzyme system of *Achromobactor sp.*, UH_3 metabolizing γ-ABA requires specifically bivalent but not trivalent iron.

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* This was presented at the 19th Meeting of the Botanical Society of Japan at Kyoto, October, 1954.

** We are indebted to Dr. Kazuo Satake, the Institute of Chemistry, Faculty of Science, University of Tokyo, for supply of the bacterial strain, *Achromobacter sp.*, UH_3.

EXPERIMENTAL

Materials and Methods

Bacterial Suspension—The bacterial culture medium contained 5 g. of bouillon, 10 g. of peptone, 2.5 g. of NaCl, and 30 g. of agar per liter. After cultivation for 36–48 hours at 30°, the cells of *Achromobacter sp.* (UH_3) were harvested by centrifugation, washed three times with distilled water, and suspended in $M/30$ Na_2HPO_4-KH_2PO_4 buffer of pH. 7.0. This suspension was used for all manometric experiments. The concentration of the suspension was determined by the Kjeldahl's measurement of nitrogen.

γ-Aminobutyric Acid—Prepared by a modification of the Tafel's method (*1*).

Metal Salts Solutions—Metal salts such as $FeCl_2$, $Co(NO_3)_2$ $Ni(NO_3)_2$, $CuSO_4$, $AgNO_3$, $MnSO_4$, and $ZnSO_4$, were used. The effects of anions were negligible in the case of the present investigations. All agents were the purest of commercial products.

Chelating Agent Solutions—Agents such as DDC, α,α'-dipyridyl, 8-hydroxyquinoline (Oxine), *o*-phenanthroline, ethylendiamine tetraacetate (EDTA), and nitroso-R-Salt (Na α-nitroso-β-naphthol-3,6-disulfonate), were dissolved in distilled water, and adjusted to pH 7.0.

Measurement Procedures—Measurement of the reaction process was carried out by using the Warburg's manometer at 30°. The reaction mixture contained $M/30$ of phosphate buffer, pH. 7.0, 4 μM of γ-ABA, and the bacterial suspension in a total volume of 2.0 ml. In the center well, 0.1 ml. of 20% KOH was placed, which absorbed CO_2 produced during the reaction. By tipping the metal ions placed in the side arm, their inhibitory or recovering effect was tested.

RESULTS

Inhibitions with DDC and Other Chelating Agents—As will be described in details in following reports, *Achromobacter sp.*, UH$_2$ is able to metabolize γ-ABA adaptively and the formation of this enzyme system is inhibited by the presence of an 'uncoupler' such as sterptomycin, 2,4-DNP, *etc.* The bacteria behaved similarly towards DDC, as shown in Fig. 1.

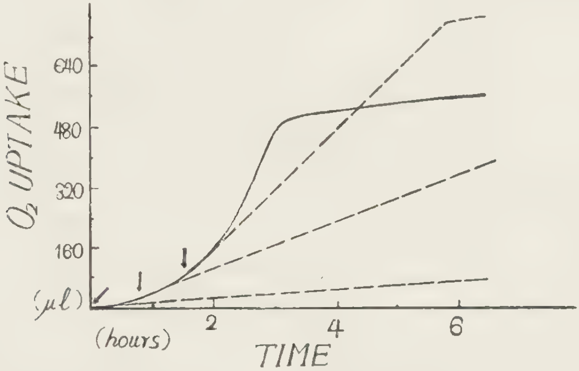


FIG. 1. Effects of DDC on the enzyme formation.

Each flask contained 0.1 ml. of $M/10$ γ-ABA, 1.3 ml. of $M/30$ Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, and 0.5 ml. of bacterial suspension. KOH was placed in the center well. Reactions were carried out at 30° for 6 hours, and O_2 uptake (μl.) was followed manometrically. Solid line, no addition of DDC; dotted lines addition of 1 μM DDC at 0, 45, or 90 minutes, as indicated by arrows.

If DDC is added to the reaction medium at 0, 45, or 90 minutes, as will be seen in Fig. 1, the bacterial cells lose the ability of enzyme formation, as soon as they contact with the agent. Among many substances tested, only an excess of Fe^{++} was able to offset completely the DDC inhibition. When Fe^{+++} is added instead of Fe^{++}, the recovery becomes very slow, but addition of vitamin C subsequent to Fe^{+++} shortens this interval, a finding interpretable in terms of the reductive formation of Fe^{++} from the latter by the action of the former. (Fig. 2).

When Fe ion is added to DDC, it becomes colored, owing to the formation of a DDC-Fe complex. Hence, it follows that DDC, forming a complex with Fe ion in the medium, depletes Fe^{++} needed by bacterial cells, and they produce the enzyme system only when an excess of Fe^{++} is added. So, it seems reasonable to conclude that Fe^{++} is essential to the enzyme formation, but not to the process of γ-ABA oxidation itself, because the latter is unaffected after the addition of DDC. In the

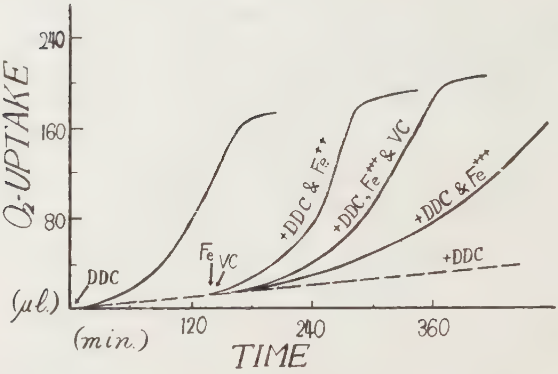


Fig. 2. Recovery from DDC inhibition with Fe ion.

Experimental conditions are the same as described in Fig. 1, except that the concentration of γ-ABA was varied to $M/25$. Final concentrations of DDC, Fe ion, and vitamin C were $5\times10^{-4}M$, $1.5\times10^{-3}\,M$, and $5\times10^{-3}\,M$, which were added at 0, 135, and 140 minutes, respectively.

final concentration of $2\times10^{-3}\,M$, DDC itself has no effect on the bacteria.

It is expected that chelating agents, having the following qualities, may possess the same actions as DDC.

(1) The agent itself is not inhibitory to the bacteria.

(2) The complex formed is not inhibitory.

(3) Under the physiological conditions (pH, temp., concentration, *etc.*) it has the ability enough to form the complex.

(4) It is soluble in water.

(5) Under the above conditions, it does not decompose.

Among many chelating agents, EDTA, *o*-phenanthroline, α,α'-dipyridyl, and Oxine were shown to have the same effect as DDC.

Inhibition by Heavy Metal Ions—Generally speaking, metal ions have various effects on the bacteria. In the case of the same ions their effects depend on their concentrations and experimental conditions for example, temperature, pH., *etc.* In our experimental system, that is, the adaptive formation of the enzyme system oxidizing γ-ABA, metal ions were used in a concentration ranged from $2\times10^{-3}\,M$ to $5\times10^{-5}\,M$.

In their effect on the bacteria, metal ions are classified into the following three groups:

Group I: Not effective......Fe^{++}, Fe^{+++}, Mn^{++}, and Zn^{++}.

Group II: Inhibitory to the enzyme formation......Co^{++}, and Ni^{++}.

Group III: Inhibitory to O_2 uptake......Ag^{+}, Hg^{++}, Cu^{++}, *etc.*

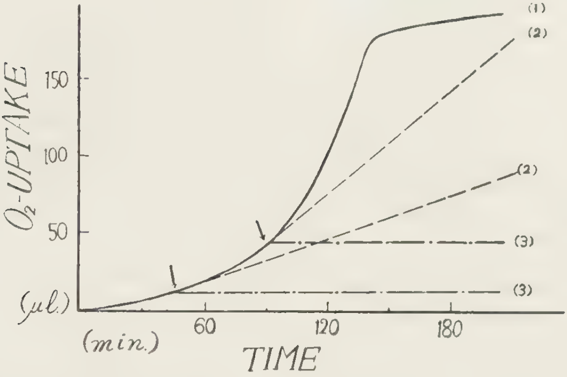


FIG. 3. Effects of metal ions on the enzyme formation.

Experimental conditions are the same as described in Fig. 2. 0.1 ml. of $M/100$ single solution of various metal salts was added at 45, or 90 minutes. Curve (1), control or added metal ions of Group I; Curve (2), Group II; Curve (3), Group III. The arrows indicated the additions of metal ions.

In the experiments illustrated in Fig. 3, metals were tipped at 45 or 90 minutes. In the case of metal ions of Group I, the curve of O_2 uptake is not affected; in Group II, the curve becomes straight owing to the cessation of enzyme formation; and in Group III, O_2 uptake also stops completely as well as the enzyme formation. Although the mechanism of these metal inhibition is yet obscure, it may be possible to find a clue to the analysis of the mechanisms by devising a method of recovering the inhibitions by metals.

Some suggestions will be given by the following facts; by adding Fe^{++} to the bacterial suspension preincubated with Co^{++}, which has the inhibitory effect of the Group II type in the final concentration of $5 \times 10^{-5}\ M$, no recovery is observed. However, when Fe^{++} and Co^{++} are added to the suspension at a time, in the final concentration of $1.5 \times 10^{-3}\ M$ and $5 \times 10^{-5}\ M$ respectively, the inhibitory effect of Co^{++} becomes negligible.

These facts may be explained on the assumption that a site specific to the essential Fe^{++} is formed in some processes of the enzyme formation and Co^{++} blocks the site instead of Fe^{++}, when the bacterial cells are preincubated with Co^{++}, but when Co^{++} is added with excess Fe^{++} at the same time, Co^{++} cannot enter the site, because overcoming by the excess Fe^{++}. So it is reasonable to conclude that Co^{++} inhibition is competitive with Fe^{++}.

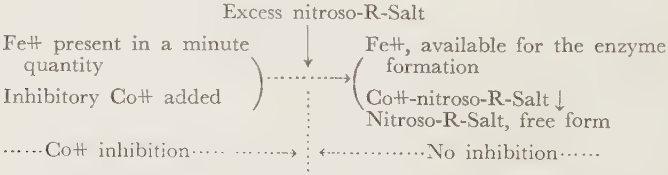
Recovery from the Metal Ion Inhibition—In the past, many methods to recover from the inhibitions by heavy metal ions in enzyme reactions and in living bodies have been devised by Bersin (*2*), Barron *et al.* (*3*), and Stockin *et al.* (*4*), using H_2S, *p*-chloromercuribenzoate, and BAL respectively. These agents, however, are not suitable for use in experiments with living cells, because they are not convenient in procedures and also have effects other than combining with the metal ions.

Though some of the inhibitions by metal ions can be eliminated by dialysis, it takes a fairly long time. There being the possibility that the bacteria in contact with such ions for a long period may suffer some secondary alteration, it is desirable that the period of recovery is as short as possible.

In the present investigations, some other methods of recovery are used.

(1) Recovery from Co^{++} inhibition with nitroso-R-salt: In order to remove Co^{++} occupying Fe site, and to place the Fe^{++} present in a minute quantity into its own site, chelating agents are required, which have a larger affinity to Co^{++} enough to drive it out of the Fe^{++} site. Nitroso-R-salt is the most suitable for this purpose, because it has a specific affinity to Co^{++}, and not to Fe^{++} under the present conditions.

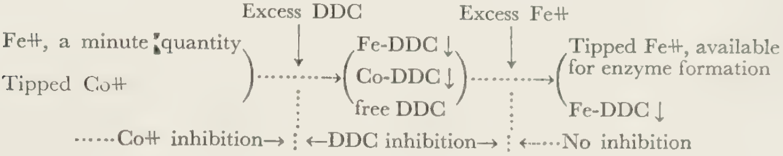
This experiment is carried out according to the following scheme;



When $5 \times 10^{-4}\ M$ of nitroso -R- Salt is added to the bacterial suspension preincubated with $5 \times 10^{-5}\ M$ of Co^{++}, Co^{++} inhibition recovers gradually. This may be the result of Fe^{++} entering into its own site and beginning the formation of the enzyme system.

In the case of Ni^{++} inhibition, anthranilic acid instead of nitroso-R-Salt is used. The result is the same as that obtained in the case of Co^{++}.

(2) Recovery from Co^{++} inhibition with DDC and Fe^{++}: Most of the agents capable of chelating with Co^{++} and Ni^{++} also have affinity to Fe^{++}. DDC is one of these. Use of DDC instead of nitroso R-Salt will change the scheme of the procedure to the following:



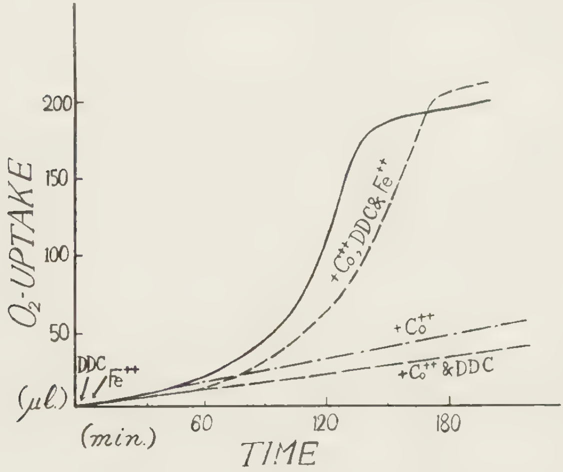


FIG. 4. Recovery from Co^{++} inhibition with DDC and Fe^{++}.

Experimental conditions are the same as shown in Fig. 2. After preincubation with 5×10^{-5} *M* of Co^{++}, 5×10^{-4} *M* of DDC and 1.5×10^{-3} *M* of Fe^{++} were added at 0, and 5 minutes respectively. The solid line is control.

In the experiment shown in Fig. 4, after preincubation with 5×10^{-5} *M* of Co^{++}, 5×10^{-4} *M* of DDC was tipped into the suspension. By this treatment Co^{++} inhibition may be replaced by DDC inhibition, but the replacement cannot be observed from the manometry of O_2 uptake. As soon as 1.5×10^{-3} *M* of Fe^{++} is added to the suspension, O_2 uptake gradually increases, indicating the beginning of the enzyme formation.

In the case of Ni++, this procedure is also applicable.

Hence it should be emphasized that the application of chelating agents is capable of recovering the enzyme activity, without dialysis, from the inhibition by some heavy metal ions.

(3) Inhibitions by the metals of Group III : It has not been able to find methods to revoer from inhibitions by Group III metals.

DISCUSSION

Reactions such as enzyme formation are very complex processes, so that even if only one step in the reactions is stopped, all reactions will be out of order and the final results, that is, the enzyme formation, will also be stopped. If only the final result is observed in experiments, many agents whose action mechanism is different, show the same result. By using the agents whose inhibition mechanism is clearly known, it will be possible to know which reaction is attacked.

Observation by the Warburg manometry showed that the bacterial cells of *Achromobacter sp.*, UH_3, suffer the same effect with DDC as with streptomycin or 2,4-dinitrophenol. From this result, it is conceivable that DDC does not belong to the ' uncouplers ', because the action of the former is only explained as a chelation with Fe++ essential to the enzyme formation, resulting in the production of the Fe++-free state, as proved in the present report.

If DDC and other chelating agents are used more systematically, the analysis of the systems concerning metal ions and the replacement of inhibitory metal ions will become easier. Chelating agents are more convenient to use than H_2S, BAL, or dialysis.

One of the first attempts in this direction was made by Ames *et al.* in 1946 (*5*). They explained how Fe++-and Cu++-proteins play their roles in the enzyme systems involving cytochrome-c, by analyzing the inhibition of those systems with many chelating agents. Since then, few experiments have been made, but such trials, if they are more systematized and extended, will serve to elucidate the mechanism of inhibition related to their enzyme system.

SUMMARY

1. The present experiments have shown that both the uncouplers and diethyl dithiocarbamate (DDC) known as a chelating agent, have the same actions in phenotype, though the mechanism of their inhibitions

is quite different. DDC exerts its inhibiting action through the appearance of Fe^{++}-free state; EDTA, Oxine, α,α'-dipyridyl, and phenanthroline, also have the same effect.

2. The adaptive formation of the enzyme system metabolizing γ-ABA of *Achromobacter sp.* UH_3 requires Fe^{++}.

3. Some of the metal ions inhibit the enzyme formation and O_2 uptake. Co^{++} and Ni^{++} show inhibitions competitive to Fe^{++}. Ag^{+}, Hg^{+}, Hg^{++}, and Cu^{++} stop O_2 uptake in the process oxidizing γ-ABA completely.

4. By using suitable chelating agents, some inhibitions by these metal ions can be easily eliminated.

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COPPER-RESISTANCE OF MYCOBACTERIUM TUBERCULOSIS AVIUM

I. THE INFLUENCES OF COPPER ION ON THE BACTERIAL GROWTH AND ON THE ACTIVITY OF SOME HIGHLY PURIFIED DEHYDROGENASES*

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(Received for publication, May 16, 1955)

It has been reported by Sher and Sweany that Cu^{++} is toxic for *Mycobacterium tuberculosis* (*1*). On the other hand, Pratt found that the growth inhibitory action of Cu^{++} for *Mycobacterium phlei* is neutralized by adding α-amino acids or citric acid to the culture medium, but not fumaric, succinic, α-ketoglutaric, acetic or lactic acid ; that results from the common property of these compounds to detoxify Cu^{++} (*2*, *3*).

In this paper, we describe that there exist fine differences between some substances, especially glucose and malate, in their abilities forming chelate compounds with Cu^{++} and neutralizing growth inhibitory action of Cu^{++} to tubercle bacilli.

MATERIAL AND METHODS

Bacterial Strain—Mycobacterium tuberculosis avium, Shibata strain, was used. The copper-resistant substrain was obtained by successive inoculation of the parent strain on a culture medium to which copper sulfate was added. In final concentration of copper sulfate added, 5×10^{-4} *M* remarkably and 8×10^{-4} *M* completely inhibited the growth of the parent strain, but 3×10^{-3} *M.* did not influence the growth of the resistant substrain.

Culture Medium and Conditions—Glycerol bouillon agar (pH 7.0) was used as the standard culture medium, which contained 10 g. of peptone, 2.5 g. of bouillon , 2.5 g. of sodium chloride, 40 g. of glycerol and 30 g. of agar per liter of distilled water. Copper sulfate was utilized as copper ion ; the effect of sulfate ion was checked with sodium sulfate to prove ineffective to the bacterial growth even in higher concentration.

The incubating temperature was 37°.

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* This paper was presented at the meeting of the Botanical Society of Japan at Kyoto, October 1954.

Preparation of the Cell Suspension—Refer previous report (*4*).

Oxidations by the Cell Suspension—Refer previous report (*4*).

Preparation of the Cell-free Extract—The grown cells were washed three times with distilled water, ground with dry ice to freezing powder, thawed at 30°, kept under an equal volume of $M/10$ phosphate buffer solution (pH 6.0) or distilled water overnight in an refrigerator, and then centrifuged at 14,000 r.p.m. (Servall Centrifuge) (*5*). The supernatent was used as the cell-free enzyme extract.

Oxidation by the Cell-free Enzyme Extract—Measurements were made in a Warburg's manometric apparatus at 30°. The extract could oxidize malate or fumarate without addition of hydrogen carrier such as methylene blue or cytochrome c as well as the preparation by the ordinary quartz-powder method could (*6*), but not citrate nor *cis*-aconitate without addition of methylene blue, though the oxidations of the latter two were accelerated by Mn^{++}.

Diphosphopyridine Nucleotide (DPN)—This (96 per cent purity) was prepared from bakers' yeast according to the method of Okunuki, Hagihara, Sekuzu, Nozaki and Nakai (*7*).

Triphosphopyridine Nucleotide—S. Naono* kindly supplied a preparation of 80 per cent purity.

Measurement of Iso-Citric Dehydrogenase—The cell-free enzyme extract was dialysed against distilled water during two days, which could reduce TPN, but not DPN when citrate was added as the substrate. The reduction of cofactors was measured at 340 mμ by Shimazu spectrophotometer at a room temperature.

Preparations and Estimations of Enzymes—Glyceraldehyde phosphate dehydrogenase was prepared from rabbit muscle according to the method of Cori, Slein and Cori (*8*) and recrystallized four times. The final recrystallization was carried out in the presence of 0.03 M cysteine and small amount of ethylenediamine tetraacetate. For the measurement of the activity, the crystals were dissolved in 0.01 M arsenate buffer solution (pH 7.6) and dialysed against the same buffer solution overnight in an ice-box. Glyceraldehyde was used as substrate. Alcohol dehydrogenase was highly purified from bakers' yeast by the method of Racker (*9*). Ethanol was used as substrate. The activities of both enzymes were measured by persuing the reduction of DPN.

RESULTS

Influences of Various Substances on the Copper Ion Inhibition of the Growth of Mycobacterium tuberculosis avium—When various substances were added to the standard culture medium containing copper sulfate in the final concentration of 8×10^{-4} M, appearances of growth inhibiting action of Cu^{++} changed as shown in Table I.

Aspartate, oxalate, citrate, ammonium citrate, glucose, mannitol markedly neutralized the growth inhibitory action of Cu^{++}. Aspartate is well known to form a copper complex sparingly soluble in water. Urease, readily inactivated by heavy metal ions, is protected from these poisonous power by the addition of protein, amino acid, or many other substances (*10,11,12*). Therefore, it seems likely that at least the protection by most of the additions described in the Table resulted from

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* Institute for Microbial Diseases, University of Osaka.

TABLE I

Influences of Various Substances Added to the Culture Medium on the Copper Ion Inhibition of the Growth of Mycobacterium tuberculosis avium, and Oxidations of the Additions by the Cell Suspension and by the Cell-free Extract

The culture medium contained each of the additional substance ($M/2$) together with and without copper sulfate. Final concentration of copper, 8×10^{-4} M. The parent strain was incubated at 37° for 5 days.

* Refer previous reports (*13, 14, 15*).

(STM) means the remarkable inhibition of oxidation by streptomycin added in the final concentration of 10 γ/ml.

Additions	State of growth		Oxidation	
	Culture medium			
	Without copper	With copper	By cell suspension	By enzyme preparation
Nil	⧻	−		
Aspartate	⧻	⧻	+(STM)	
Glutamate	⧻	⧺	+(STM)	
Acetate	⧻	+	+*	
Oxalate	⧻	⧻	+	+
Malate	⧻	+	+(STM)*	+
Succinate	⧻	+	+(STN)*	
Malonate	⧻	⧺	+(STM)*	
Citrate	⧻	⧻	−	+
Ammonium citrate	⧻	⧻	−	+
Glucose	⧻	⧻	+	
Mannitol	⧻	⧻	+	
Arabic gum	⧻	+		
Ammonium sulfate	⧻	±		

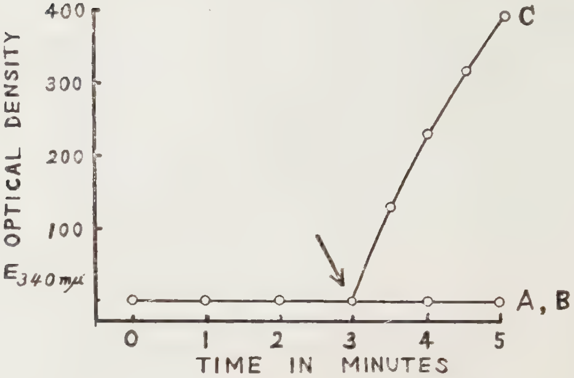

FIG. 1. Reduction of TPN by the cell-free enzyme extract in the presence of citrate

Contents of cuvette : The dialyzed enzyme extract (diluted 8 times as much as the extract used in manometric measurement) 0.5 ml., $M/10$ phosphate buffer solution (pH 7.0) 1.0 ml., citrate aq. ($M/50$) 0.5 ml., $MgSO_4$ aq. ($M/100$) 0.2 ml., and DPN (2 mg./ml.) or TPN (2 mg./ml.) 0.5 ml. The total volume was adjusted to 3.5 ml. with distilled water. Reacted at room temperature (28°). A, DPN or TPN without citrate ; B, DPN+citrate ; C, TPN added at the arrow point on Curve B.

their formation of chelate compounds with Cu^{++}.

The copper-resistant property acquired by successive inoculation of the parent strain on the standard culture medium containing copper sulfate was heritable over ten generations on the medium, not containing copper sulfate. However, the neutralization of copper inhibition by an additional substance did not result in the acquirement of copper-resistant ability, since when the parent strain well-grown on the culture medium containing aspartate or glucose as an additional substance and 10^{-3} M of copper sulfate was transplanted to the medium containing copper sulfate alone, no increase in the resistant ability could be demonstrated.

Oxidations by the Cell Suspension—The suspension could remarkably oxidize aspartate, glutamate, oxalate, glucose and mannitol, but not citrate. The oxidations of glucose and oxalate were not influenced in the presence of streptomycin (100 γ/ml.) at all, even by utilization of any aged cell suspension in contrast to the oxidation of succinate, fumarate or malte (*13,14*).

Oxidations by the Cell-free Eyzyme Extract—As shown in Fig. 1, the dialysed enzyme extract could not reduce DPN in the presence of citrate. However, when TPN was added on the way, its reduction proceeded swiftly. This extract did not oxidize manometrically succinate and α-ketoglutarate in the experimental conditions, in which citrate, oxalate, fumarate and malate were markedly oxidized.

Influences of Copper Ion on the Activities of Highly Purified Alcohol Dehydrogenase and of Crystalline Glyceraldehyde Phosphate Dehydrogenase—The activities of alcohol dehydrogenase and of glyceraldehyde phosphate dehydrogenase were markedly inactivated in the presence of low concentration of copper ion as shown in Figs. 2 and 3. When aspartate was added to the reaction mixture, the inhibitory action of Cu^{++} was remarkably neutralized, although it has not direct connection with the DPN-reduction. Remaining activity of the inhibited dehydrogenases

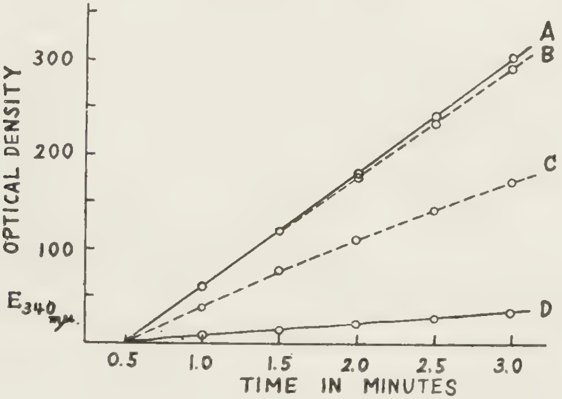

FIG. 2. Neutralizing action of aspartate against the inhibition of copper ion on the activity of highly purified alcohol dehydrogenase

The components of the reaction mixture were as follows : 2% solution of ethanol in *M*/33 pyrophosphate buffer (pH 7.0) 2.0 ml., 2 mg./ml. DPN aq. 0.5 ml., 0.5 *M* asparate 0.3 ml., copper sulfate aq. (final concn. 1.25×10^{-4} *M*) 0.3 ml., and alcohol dehydrogenase solution in *M*/100 phosphate buffer (pH 7.0) 0.5 ml. The enzyme solution was mixed at 0 time in the reaction. The reaction was carried out at room temperature. Curve A, ethanol; B, ethanol and aspartate ; C, ethanol, asparate and copper : D, alcohol and copper. The total volume of the reaction mixture was brought to 3.6 ml. with distilled water.

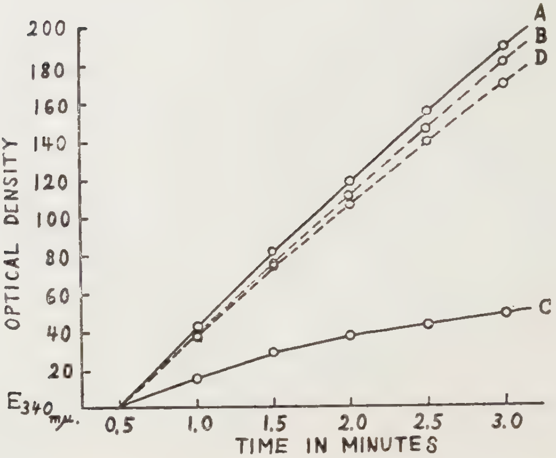

Fig. 3. Neutralizing action of aspartate against copper ion inhibition of the activity of crystalline glyceraldehyde phosphate dehydrogenase

The components of the reaction mixture were as follows : *M*/33 of pyrophosphate buffer solution (pH 9.0) containing 1 mg./ml. of glyceraldehyde, DPN aq. (2 mg./ml.) 0.5 ml., aspartate (*M*/2) 0.3 ml., copper sulfate aq. (final concn. 8.3×10^{-6} *M*) 0.3 ml., and glyceraldehyde phosphate dehydrogenase in *M*/33 of pyrophosphate buffer solution (pH 9.0) 0.5 ml. The enzyme was added at 0 time in the reaction. The reaction was carried out at room temperature. Curve A, glyceraldehyde : B, glyceraldehyde and aspatate : C, glyceraldehyde and copper : D, glyceraldehyde, copper and aspartate. The total volume of the reaction mixture was adjusted to 3.6 ml. with distilled water.

after three minutes of copper-treatment, in the presence and absence of additions, are shown in Tables II and III, expressed as per cent of the untreated one. Among the additions tested, aspartate and malate were the most and citrate was secondly effective in protecting their activities against copper ion, while none of succinate, tartarate, acetate, and glucose showed any effect to a considerable extent.

Such an neutralizing action of the additional substances seems likely to result from formation of chelate compounds of Cu^{++} and the additions, though the influence of glutamate on the activity of glyceraldehyde dehydrogenase suggests that there exists some interrelationships between the enzyme protein and glutamate as shown in Fig. 4.

TABLE II

Neutralizing Action of Various Additional Substances against the Inhibition of the Activity of Highly Purified Alcohol Dehydrogenase by Copper Ion.

The experimental conditions were the same as for Fig. 1.

Addition	Remaining activity (%)
Nil	11
Aspartate	66
Succinate	35
Glucose	19
Malate	Contamination of malic dehydrogenase

TABLE III

Neutralizing Action of the Various Additoinal Substances against the Inhibition of the Activity of Crystalline Glyceraldehyde Phosphate Dehydrogenase by Copper Ion

The experimental conditions were the same as described in Fig 2.

Addition	Remaining activity (%)		
	Final concentration of copper ion in the reaction mixture (M)		
	Expt.1 8.3×10^{-6} M	Expt. 2 8.3×10^{-5} M	Expt. 3 8.3×10^{-6} M
Nil	43	32	26
Aspartate			93
Malate	60	74	56
Succinate		47	
Citrate		59	
Acetate	43	44	
Tartarate			46
Glucose	43	42	

DISCUSSION

When inoculated successively on the medium containing copper sulfate, *Mycobacterium tuberculosis avium* can aquire copper-resistant ability, which is heritable without any decrease over ten generations on

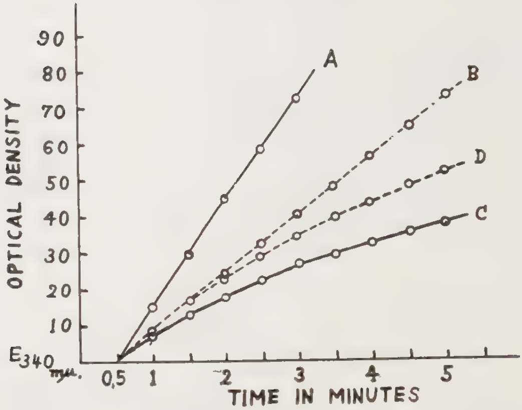

FIG. 4. Influences of glutamate on the activity of crystalline glyceraldehyde phosphate dehydrogenase in the presence and absence of copper ion

The experimental conditions were the same as shown in Fig. 2. Curve A, glyceraldehyde : B, glyceraldehyde and glutamate : C, glyceraldehyde and copper : D, glyceraldehyde, glutamate and copper. The total volume of the reaction mixture was brought to 3.6 ml. with distilled water.

the medium containing copper sulfate.

As to the parent strain, however, it can grow well only in the presence of such an additional substance as aspartate or the like, on the medium of the copper concentration otherwise enough to prevent growth of the parent strain. This does not mean the essential increase in the copper-resistant ability since the ability is in the same degree if determined on the medium containing copper sulfate without the additional substance. If the neutralizing actions of the additional substances result only from their formation of chelate compounds with Cu^{++}, the order of the neutralizing abilities should resemble that determined by using a highly purified alcohol dehydrogenase and crystalline glyceraldehyde phosphate dehydrogenase. But this is not the case for glucose and malate, as can be seen from Tables I, II and III. Such differences between the two experiments suggest that the neutralizing ability of the additional substances in the culture medium does not always result from their formation of chelate compounds with copper ion, effecting removal of copper ion poisonous to the microörganisms from the medium. The microoörganisms can well oxidize glucose throughout the whole cellular age. Therefore, it seems to be oxidized into some intermediate, which has a strong neutralizing action. The copper-resistant ability of this microoörganisms may mean such a heritable aquirement of a neutralizing action as is caused by the addition of glucose in the presence of copper sulfate.

SUMMARY

The influences of copper ion on the DPN-reduction of highly purified alcohol dehydrogenase extracted from bakers' yeast and of crystalline glyceraldehyde phosphate dehydrogenase extracted from rabbit muscle were observed in the presence and absence of various additional substances in the reaction mixture. The neutralizing action of the additions tested against the copper inhibition was estimated as follows: Aspartate and malate were strong, citrate medium, and succinate, tartarate, acetate and glucose weak.

When some of the substances described above were added to the culture medium, *Mycobacterium tuberculosis avium* could grow well in the presence of copper in a concentration enough to inhibit the growth in the medium without the additions. This phenomenon did not follow the essential increase in the copper-resistant ability. The grade of the neutralizing power was follows: Aspartate, oxalate, citrate, ammonium citrate, glucose, and mannitol were strong, glutamate and malonate medium, acetate, malate, succinate, arabic gum, and ammonium sulfate weak. Namely, the influences of glucose and malate ran counter to each other in the two experiments. On the basis of such differences, possible explanation on the increase in the copper-resistant ability of the microoörganisms was discussed.

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COPPER-RESISTANCE OF MYCOBACTERIUM TUBERCULOSIS AVIUM

II. THE INFLUENCE OF COPPER ION ON THE RESPIRATION OF THE PARENT CELLS AND COPPER-RESISTANT CELLS*

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(Received for publication, May 16, 1955)

In the previous report (*1*), it has been shown that there exists a possibility of glucose to be oxidized by tubercle bacilli into some other substance, which can easily form chelate compounds with copper ion, and such a reactions may serve the increase in the copper-resistant ability, since the protective power of glucose against the copper inhibition of the growth is remarkably stronger than that of malate, while glucose is by far less potent than malat in removing this inhibition by the formation of chelate compounds with copper ion.

The present paper deals with the permeability of copper ion into the plasma and the influence of copper ion on the respirations of the microörganisms. The glucose oxidation of the parent cells was certified to be extremely accelerated in the presence of a minimum concentration of copper ion to interfere with the growth, but the malate oxidation to be inhibited.

MATERIAL AND METHODS

Bacterial Strain—Mycobacterium tuberculosis avium, Shibata strain, was used. The copper-resistant substrain was obtained by successive inoculation (*1*).

Culture Medium and Conditions—Refer the previous report (*1*).

Preparation of the Living Cell Suspension—Refer the previous report (*2*).

Estimation of Enzyme Activity—This was carried out by Warburg's manometric apparatus. The experimental conditions were essentially the same as those described in the previous report (*2*).

Estimation of Copper Ion—The copper ion contained in the cells was estimated by the method of Kikkawa and Ogita (*3*). The grown cells were harvested, washed three times with distilled water, dried at 100° for 12 hours, ground into a powder, and weighed to 100 mg. Ashing of the weighed powder was carried out with 5 ml. of HNO_3 and then with 2 ml. of H_2SO_4, frequently using one drop of 30 per cent H_2O_2.

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* The compendium of this article was presented at the meeting of Botanical Society of Japan at Kyoto in October, 1954.

RESULTS

Comparison of the Copper Ion Contained in the Parent Cells and in the Copper-resistant Cells—The copper-resistant substrain was grown on the culture medium (without addition of copper sulfate) prior to inoculation for the estimation of copper. The copper culture medium contained copper sulfate to a concentration (10^{-4} *M*) which does not interfere with the growth of the parent strain as well as that of the resistant substrain.

TABLE I

Comparison of Copper Ion Content in the Parent Cells and in the Copper-resistant Cells

The parent cells and the copper-resistant cells were grown on the standard culture medium (without addition of copper sulfate) and on the copper culture medium (10^{-4} *M* in final concentration).

Days incubated	Copper ion content in the cells, γ/100 mg. of dry weight			
	Grown on standard culture medium		Grown on copper (10^{-4} *M*) culture medium	
	Parent cells	Resistant cells	Parent cells	Resistant cells
3	16	12	42	29
5	14	12	—	—
9	10	9	43	30
20	13	9	60	30

As shown in Table I, when inoculated on a standard culture medium, the copper ion content of the parent cells and that of the copper-resistant ones were almost the same. However, when inoculated on a copper culture medium, the parent cells was estimated to contain two to three times as much copper ion as the cells inoculated on the standard culture medium. Such increase in copper content was demonstrated as well with the copper-resistant cells, but the grade was lower in the resistant cells than in the parent cells. From this result, it is suggested that copper ion is more permeable into the latter than into the former.

Comparisons of the Influence of Copper Ion on the Respirations of the Parent Cells and of the Copper-resistant Cells—The oxidations of malate by the parent cells and by the resistant cells were inhibited in the presence of copper ion, as shown in Figs. 1 and 2. The concentration of copper ion inhibitory to the oxidation of malate by the cells increased in parallel with a rise of their copper-resistant ability. The oxidations of succinate and fumarate suffered influence almost similar to those of malate.

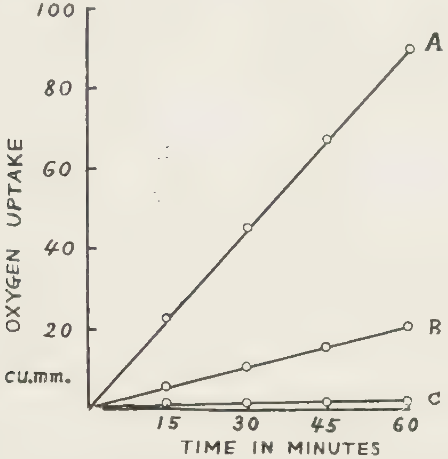

FIG. 1. Inhibiting effect of copper ion on the oxidation of malate by the parent cells.

Incubation of 5 days. Dry weight of the cell suspension, 16 mg./ml. Before addition of the substrate, the cell suspension was preincubated with copper ion for 15 minutes. A, 8 μM of malate : B, 8 μM of malate +copper sulfate, 5×10^{-4} *M* : C, 8 μM of malate+copper sulfate, 10^{-3} *M*.

The glucose oxidation was also influenced by copper ion, but the mode of action of copper ion on the glucose oxidation was different from that on the malate oxidation. In the oxidation of glucose by the parent cells, the amount of oxygen consumed and that of carbon dioxide evolved in the presence of 10^{-3} *M* copper ion were more than twice as much as in its absence, as shown in Figs. 3 and 4, while with the resistant cells the oxidation was little influenced, as shown in Fig. 5. On the other hand, the oxygen consumed by the glucose oxidation in the absence of

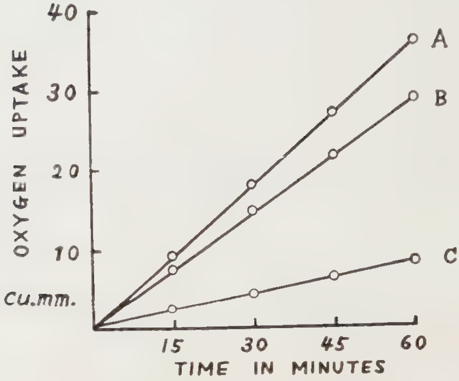

FIG. 2. Inhibiting effect of copper ion on the oxidation of malate by the copper-resistant cells.

Dry weight of the cell suspension, 12 mg./ml. The other experimental conditions were the same as for Fig. 1.

A, 8 μM of malate : B, 8 μM of malate+copper sulfate, 5×10^{-4} *M* : C, 8 μM of malate+copper sulfate, 10^{-3} *M*.

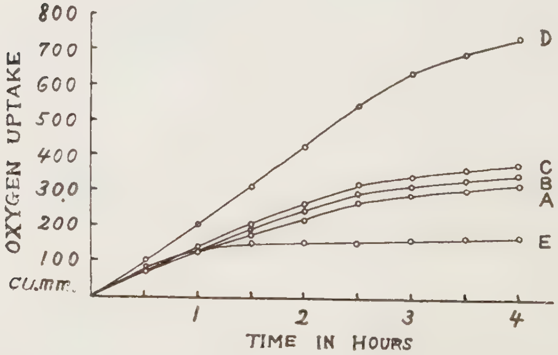

FIG. 3. Influence of copper ion on the oxidation of glucose by the parent cells.

Dry weight of the cell suspension, 18 ml./ml. The other experimental conditions were the same as for Fig. 1. A, 8 μM of glucose : B, 8 μM of glucose+copper sulfate, 10^{-5} *M* : C, 8 μM of glucose+copper sulfate, 10^{-4} *M* : D, 8 μM of glucose+copper sulfate, 10^{-3} *M* : E, 8 μM of glucose+copper sulfate, 10^{-2} *M*.

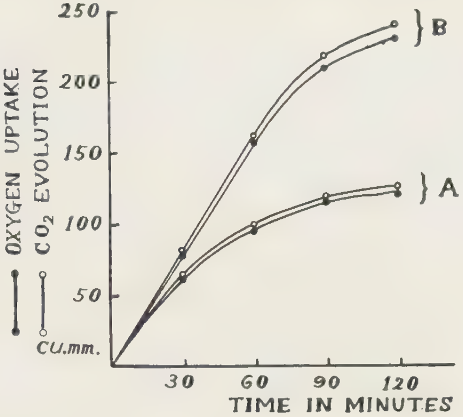

FIG. 4. Influence of copper ion on the carbon dioxide evolution in the glucose oxidation by the parent cells.

The experimental conditions were the same as for Fig. 3. A, 4 μM of glucose : B, 4 μM of glucose+copper sulfate, 10^{-3} *M*.

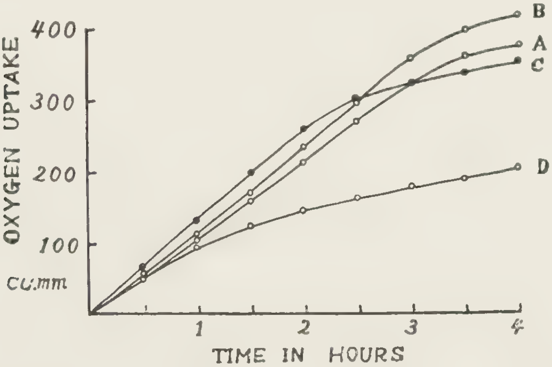

FIG. 5. Influence of copper ion on the glucose oxidation by the copper-resistant cells.

Dry weight of the cell suspension, 17 mg./ml. The other experimental conditions were the same as for Fig. 3. A, 8 μM of glucose : B, 8 μM of glucose+copper sulfate, 10^{-4} *M* : C, 8 μM of glucose+copper sulfate, 10^{-3} *M* : D, 8 μM of glucose+copper sulfate, 10^{-2} *M*.

copper ion was approximately three atoms per mole of glucose in the parent cells (Fig. 6) and four atoms in the resistant cells (Fig. 7).

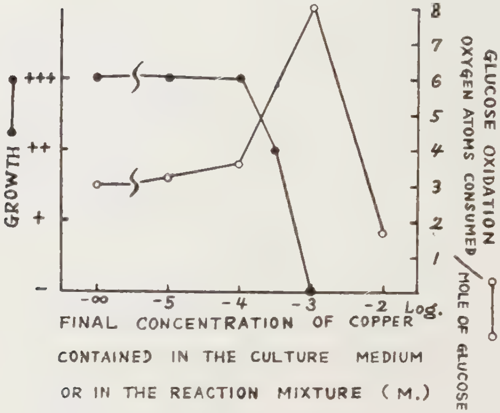

FIG. 6. The influences of copper ion on the growth of the parent cells and on the oxygen atoms per mole of glucose consumed by the cell suspension.

The growing state of the cells was judged after 7 days culture. The oxygen atoms consumed was calculated at the time when the glucose oxidation arrived at an equillibrium (Refer Fig. 3).

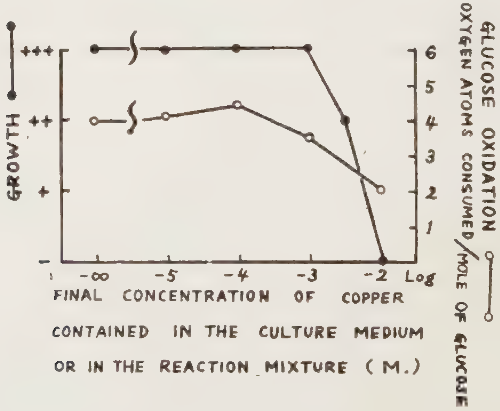

FIG. 7. The influences of copper ion on the growth of the copper-risistant cells and on the oxygen atoms per mole of glucose consumed by the cell suspension.

As to the experimental conditions, refer Figs. 5 and 6.

DISCUSSION

The results show that copper ion is more difficult to penetrate into the plasm of the copper-resistant substrain than into that of the parent strain of tubercle bacilli. It seems unlikely, however, that the copper-resistant ability is explainable simply through alteration of the plasmic membrane against the penetration of copper ion, since a remarkable amount of copper ion penetrates into the plasma of the resistant cells.

The living cell suspension can oxidize malate, fumarate, succinate, glucose, glycerol and acetate. The oxidations of the former three by the parent cells are remarkably inhibited by copper ion in a similar concentration as to interfere with their growth, but the oxidations by the resistant cells are a higher concentration as well as their growth. The glucose oxidation by the parent cell suspension consumes approximately three oxygen atoms per mole of glucose and, in the presence of copper ion, it is accelerated over twice as much. The glucose oxidation by the copper-resistant cell suspension consumes approximately four atoms per mole of glucose and it is hardly influenced in the presence of the same concentration of copper ion. If the copper-resistant ability is induced only by the change of permeability, glucose oxidation by the copper-resistant cells should be also accelerated in the same manner as that by the parent cells in the presence of a higher concentration of copper ion, but such acceleration can not be demonstrated with the copper-resistant cells, even in the presence of any concentration of copper ion tested.

These facts suggest that the copper-resistant ability of tubercle bacilli is caused at least by both of the alteration of the plasmic permeability against copper ion and a certain change of the intracellular metabolism.

SUMMARY

With *Mycobacterium tuberculosis avium*, penetration of copper ion into the plasm and the influences of copper ion on the respiration were observed.

1. When the microörganisms grow on a standard culture medium, the parent and the copper-resistant cells are almost the same in their copper content. When they grow on a copper culture medium, the parent cells contain more copper ion than the resistant ones.

2. The oxidations of malate, fumarate and succinate by the parent cells are remarkably inhibited by copper ion in a similar concentration as to interfere with their growth, but the oxidations by the

resistant cells in a higher concentration as well as their growth.

3. The oxidation of glucose by the parent cells is markedly accelerated in the presence of copper ion while that by the resistant cells scarcely not in any concentration of copper ion tested.

These results lead to the supposition, that the copper-resistant ability of tubercle bacilli is induced by the alteration of the plasmic permeability against copper ion and by a certain change of the intracellular metabolism.

This work was in part supported by the Grant in Aid for Experimental Scientific Research from the Ministry of Education.

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THE SYNTHESIS OF STERO-BILE ACID.

XIII. THE PARTIAL SYNTHESIS OF HOMOCHOLANIC ACID, BISHOMOCHOLANIC ACID AND 25-ETHYL-BISHOMOCHOLANIC ACID

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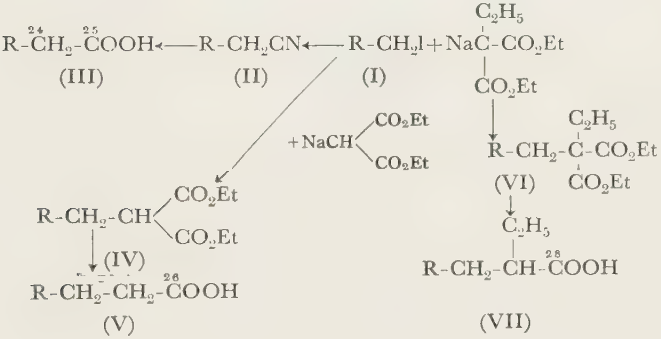
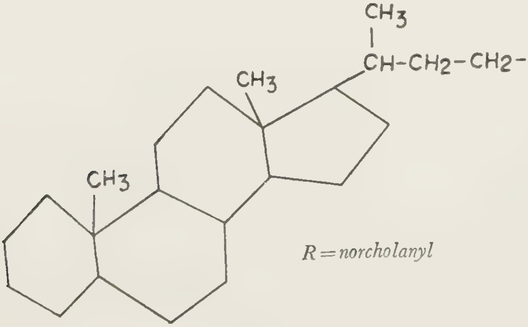
(Received for publication, May 16, 1955)

In this series, Kazuno and co-workers (*1*) have already synthesized the various stero-bile acids or bile steroids having more number of carbon atoms than the normal bile acid. A fundamental problem in studying the stero-bile salts of primitive animal is to provide proof of the nature



$R\text{-}\overset{24}{C}H_2\text{-}\overset{25}{C}OOH$
(III)
$R\text{-}CH_2CN$
(II)
$R\text{-}CH_2I$
(I)
$+NaC(C_2H_5)(CO_2Et)\text{-}CO_2Et$
$+NaCH(CO_2Et)_2$
$R\text{-}CH_2\text{-}CH(CO_2Et)_2$
(IV)
$R\text{-}CH_2\text{-}CH_2\text{-}\overset{26}{C}OOH$
(V)
$R\text{-}CH_2\text{-}C(C_2H_5)(CO_2Et)\text{-}CO_2Et$
(VI)
$R\text{-}CH_2\text{-}CH(C_2H_5)\text{-}\overset{28}{C}OOH$
(VII)



R = norcholanyl

of the carbon skeleton of bile steroids or stero-bile acids whose molecules probably contain twenty-five or twenty-nine carbon atoms. The present paper deals with the synthesis of the several stem acids of stero bile acid by the following scheme.

Cholanic acid was converted by lithium aluminium hydride reduction on the ethylester to cholan-24-ol which was converted into the corresponding iodide (I) by the Haslewood method (*2*). The iodide was condensed with KCN to give the cholan-24-nitril (II) which on hydrolysis yielded the homochloanic acid, $C_{25}H_{42}O_2$ (III). The iodide was condensed with diethyl sodiomalonate and with dietyl sodioethylmalonate to give the respective substituted malonesters (IV and VI) which on hydrolysis yielded the corresponding free acids. Decarboxylation of the former in vacuo gave bishomocholanic acid, $C_{26}H_{44}O_2$ (V) and the latter gave 25-ethylbishomocholanic acid, $C_{28}H_{48}O_2$ (VII). The homocholanic acid and bishomocholanic acid were respectively stem acids of homocholic acid (*3*) and bishomocholic acid (*4*) which already had been synthesized by Kazuno and co-workers (*1*).

EXPERIMENTAL

24-Iodocholane (*I*)—A procedure being identical with the method described by Haslewood, with cholane-24-ol (15 g.), I_2 (21 g.) and red P (1.7 g.) yielded, after chromatography, crystals (15 g.) which on recrystallization from acetone gave 24-iodocholane as long white needles, m.p. 100–101°.

Analysis. Calcd. for $C_{24}H_{41}I$: C 63.15, H 8.99
Found : C 63.21, H 8.82

Cholane-24-nitril (*II*)—*24*-Iodocholane (1 g.) was refluxed for 48 hour with KCN (1 g.) in ethanol-water (155 ml. : 13.5 ml.). Ethanol was evaporated from the reaction mixture to give a yellow crystalline solid which was recrystallized from ethanol. This yielded white crystals (900 mg.), needle of cholane-24-nitril, (m.p. 122–123°.)

Analysis. Calcd. for $C_{25}H_{41}N$: C 84.51, H 11.55, N 3.94
Found : C 84.42, H 11.71; N 4.05

Homocholanic Acid (*III*)—700 mg. of cholane-24-nitril and 7 g. of potassium hydroxide were dissolved in 140 ml. of ethanol-water (125 : 15), and this solution was hydrolysed on a water bath for 48 hour. Ethanol was evaporated under reduced pressure and dilution with water gave a clear solution which on acidification with HCl yielded a solid. The resulting precipitate, was extracted with ether. The ether solution was washed with water, dried and the ether was distilled off. The residue was recrystallized from acetone and then from ethanol-water. 200 mg. of needle like crystal with m.p. 102–103° were obtained.

Analysis. Calcd. for $C_{25}H_{42}O_2$: C 80.32, H 11.23
Found: C 80.50, H 11.41

25,25-Dicarbethoxy-24-homocholane (*IV*)—A mixture of Na (1 g.) and diethyl malonate (10 g.) was refluxed with 50 ml. of toluene until the formation of the sodio derivative was apparently complete. 24-Iodocholane (5 g.) dissolved in 30 ml. of dry toluene was then slowly added from a dropping funnel whilst the mixture was boiled under reflux.

After boiling for a further 6 hour, the reaction mixture was treated with water and extracted with ether. The extract, after washing with water, drying with Na_2SO_4 and evaporation, gave an oil. This oil was dissolved in petroleum ether and adsorbed on a column of Al_2O_3 (120 g.). Elution of the column with petroleum ether (1000 ml.) and then with 500 ml. of petroleum ether-benzene (80 per cent $^{v}/_{v}$), gave 1.8 g. of crystalline material, recrystallization of which from absolute alcohol gave 25, 25-dicarbethoxy-24-homocholane (m.p. 55–56°.)

Analysis. Calcd. for $C_{31}H_{52}O_4$: C 76.23, H 10.66
Found: C 76.20, H 10.83

25,25-Dicarboxy-24-homocholane—A mixture of 1.5 g. of the above ester, 23 ml. of absolute ethanol and 1.5 g. of KOH (dissolved in 2 ml. of water) was refluxed on a water bath for 2 hour. After ethanol was evaporated, dilution with water gave a clear solution which on acidification with HCl yielded a solid. The resulting precipitate, was extracted with ether. The ether solution was washed with water, dried and the ether was distilled off. The residue was recrystallized from acetone. 1.2 g. of prism like crystal with m.p. 161.5° (foaming) were obtained.

Analysis. Calcd. for $C_{27}H_{44}O_4$: C 75.00, H 10.19
Found: C 75.11, H 10.21

Bishomocholanic Acid (*V*)—One g. of the above product was heated in sublimation apparatus at 15 mm.-Hg. at about 165°, decarboxylation occurred and the product changed into brown oil. This brown oil was recrystallized from aceton. 800 mg. of bishomocholanic acid with needle like crystal (m.p. 130–131°) were obtained.

Analysis. Calcd. for $C_{26}H_{44}O_2$: C 80.04, H 11.34
Found: C 80.20, H 11.41

Analysis of methyl bishomocholanate with m.p. 61–62°.

Calcd. for $C_{27}H_{46}O_2$: C 80.60, H 11.42
Found: C 80.51, H 11.31

25-Ethyl-25,25-dicarbethoxy-24-homocholane (*VI*)—A mixture of Na (1 g.) and diethyl ethylmalonate (10 ml.) was refluxed with 50 ml. of dry toluene until the formation of the sodio derivative was apparently complete. 24-Iodocholane (5 g.) dissolved in 30 mg. of dry toluene was then slowly added from a dropping funnel whilst the mixture was boiled under refluxed. After boiling for a further 6 hour, the reaction mixture was treated with water and extracted with ether. The extract, after washing with water, drying with Na_2SO_4 and evaporation, gave an oil. This oil was dissolved in petroleum ether and chromatographed on a column of Al_2O_2. Elution of the column with petroleum ether (1000 ml.) gave 3.5 g. of crystalline material, recrystallization of which from absolute alcohol gave 25-ethyl-25, 25-dicarbethoxy-24-homocholane, m.p. 60–61°.

Analysis. Calcd. for $C_{33}H_{56}O_4$: C 76.75, H 10.85
Found : C 76.96, H 11.04

25-Ethyl-25,25-dicarboxy-24-homocholane—A mixture of 3 g. of the above ester, 46 ml. of absolute ethanol and 3 g. of KOH (dissolved in 4 ml. of water) was refluxed on a water bath for 2 hours. After ethanol was evaporated, diluted with water gave a clear solution which on acidification with HCl yielded a solid. The resulting precipitate, was extracted with ether. The ether solution was washed with water, dried and the ether was distilled off. The residue was recrystallized from acetone-petroleum ether. 1.5 g. of needle like crystal with m.p. 194-195° were obtained.

Analysis. Calcd. for $C_{29}H_{48}O_4$: C 75.65, H 10.43
Found: C 75.49, H 10.32

25-Ethyl-bishomocholanic Acid (VII)—One g. of the above product was heated in sublimation apparatus at 15 mm.-Hg. at about 200°, decarbosylation occured and the product changed into brown oil. This oil was distilled up to about 300°, forming a nearly colourless oil which was not crystallized.

Analysis. Calcd. for $C_{28}H_{48}O_2$: C 80.76, H 11.53
Found: C 80.47, H 11.76

The author wishes to express his gratitude to Prof. T. Kazuno for his kind guidance.

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The Journal of Biochemistry, Vol. 42, No. 5, 1955

STUDIES ON XANTHURENIC ACID

XII. EFFECT OF XANTHURENIC ACID ON PHOSPHORUS METABOLISM

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(Received for publication, May 26, 1955)

Since the discovery of hexose phosphate by Harden (*1*) and Young the role of phosphoric acid in carbohydrate metabolism has aroused keen interests among many research workors. Harrop and Benedict (*2*), Bollinger and Hartmann (*3*) reported the decrease of inorganic phosphate in blood following the oral as well as paraenteral administrations of glucose. Such a decrease was also observed by Cori and Cori (*4*) and by Kaplan (*5*) after the injection of insulin. It will be well imazined that in the case of diabets mellitus some disturbance in the phosphoric acid metabolism may occur.

Recently Sacks (*6*) reported that the effect of alloxan diabetes on the composition of the acid-soluble P of the liver was studied and the increase of about 50 per cent in the concentration of inorganic phosphate and the decrease of about one-third in the concentration of ATP-ADP, glucose-1-phosphate and glucose-6-phosphate were observed. As reported in foregoing series of this study, xanthurenic acid acts as a diabetogenic agent against rat (*7, 8*). It seems interesting, therefore, to investigate the effect of xanthurenic acid upon the phosphorus metabolism in corelation of diabetic symptoms in rats.

EXPERIMENT

A) Rats weighing about 200 g. were fed on the food as shown in Table I.

A number of them were injected with an aqueous solution (pH 7.2) of xanthurenic acid (synthetic, m.p. 269.5°) intraperitoneously in the dosis of 300 mg. per kg. of body weight. At the time after the injection as indicated in the graph the animals were sacrificed one by one by decapitation, and the blood sugar was determined by the King-Garner's metod (*9*), blood phosphocreatine and inorganic phosphate by the King (*10*) and Shimazono-Takahashi's method (*11*),

TABLE I

Synthetic Diet

Casein	22%
Starch	52%
Yeast	2%
Agar agar	3%
Sugar	5%
Butter	10%
McCollum salt	6%

excreted xanthurenic acid by the Glazer-Mueller's method (*12*), and excreted total creatinine by the Folin-Wu's method (*13*). The results are illustrated in Figs. 1 and 2.

It will be seen from Fig. 1 that inorganic phosphate in blood and liver showed a distinct increase reaching the maximum 2–3 hours after the injection. On the contrary the phosphocreatine in blood and liver showed a decrease (Fig. 2).

In the case of application of 300 mg./kg. xanthurenic acid dissolved olive oil two times with the interval of one day a high blood sugar level appeared after one week with corresponding increase of blood inorganic phosphate.

B) In order to investigate the behavior of phosphoric acid metabolism in the case of chronic diabetic symptoms the following experiments were carried out.

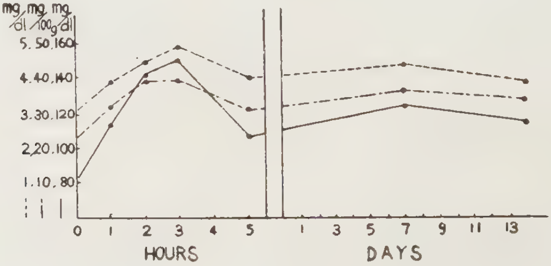

FIG. 1 (The mean value of 5 rats)

——— Blood sugar

- - - - Inorganic phosphate in blood

— - — Inorganic phosphate in liver

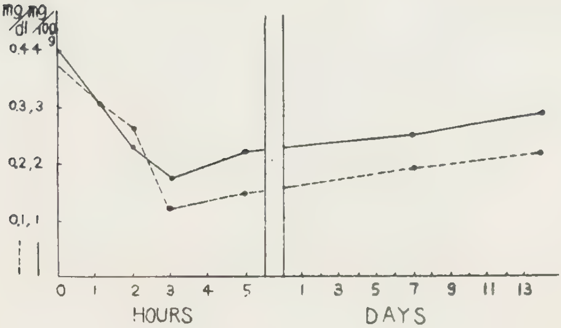

FIG. 2 (The mean value of 5 rats)
- - - - Phosphocreatine in blood
——— Phosphocreatine in liver

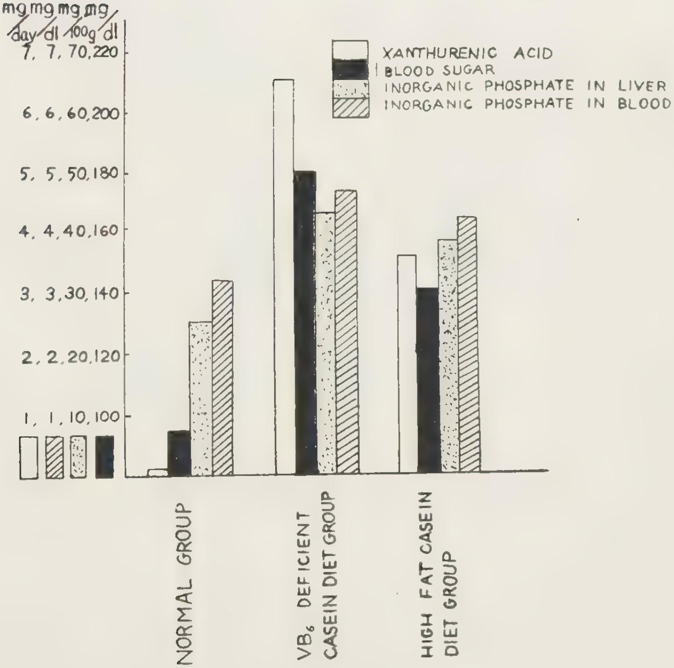

FIG. 3 (The mean value of 5 rats)

1) Rats weighing 200 g. were fed on the feed as shown in Table II. On this feed the animals received daily enough tryptophan as to excrete 1 mg. of xanthurenic acid into urine. But with the purpose to make sure the effect of xanthurenic acid, 10 mg. of tryptophan were added daily to the feed for 30 days. After 180 days where blood sugar level rose to 130–150 mg./dl. (normal 70 mg./dl.) the animals were sacrificed and inorganic phosphate and phosphocreatine in blood and liver were determined. The results showed that the former increased and the latter decreased (Figs. 3 and 4)

TABLE II

Casein Diet Containing Rich Fat

Casein	22%
Starch	28%
Medical yeast	2%
Agar agar	3%
Sugar	5%
Butter	35%
McCollum salt	2%

It was observed also that the total urine creatinine content was unmistakably increased (Fig. 5).

2) As claimed first by Leplovsky (*14*) and confirmed by our foregoing papers, vitamin B_6 plays a distinct role in the metabolism of tryptophan, its shortness leading to the increased output of xanthurenic acid.

Rats were fed on the feeds of Table III which was lack of vitamin B_6. In addition to the ingradients in the Table III 10 mg. of tryptophan were given daily. After 90 days, when the blood sugar level rose to 150 mg./dl., the animals were brought to death by decapitation and examined. The results are illustrated in Figs. 4 and 5. The inorganic phosphate in blood and liver showed an apparent increase while phosphocreatine a decrease of higher extent. Also total creatinine in urine increased (Fig. 5).

C) Effect of administration of glucose : When the animal showed a hyperglycemia due to the injection of xanthurenic acid, to high fatty foods or to lack of vitamin B_2, 2 g. of glucose per kg. were applied to the animal *per os* as 50 per cent aqueous solution, and blood inorganic phos-

TABLE III

Casein Diet Deficient in Vitamin B_6

Hammersten casein	20%	Besides these, following vitamins in each 1 g. food Vitamin B_1	3.3 γ
McCollum salt	4%	Nicotinic acid	10.0 γ
Sugar	74%	Calcium pantothenate	13.3 γ
Salad oil plus liver oil	2%	Cholin chloride	166.0 γ
		Inositol	333.0 γ
		Riboflavin	6.6 γ
		p-Aminobenzoic acid	200.0 γ

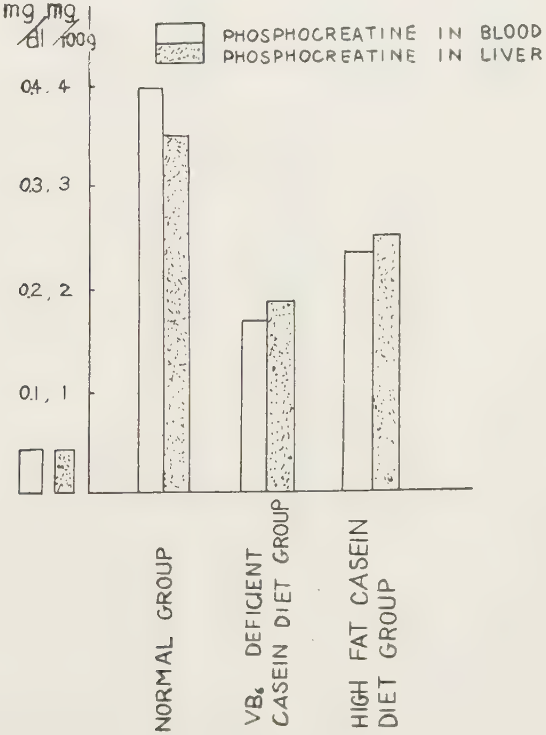

FIG. 4 (The mean value of 5 rats)

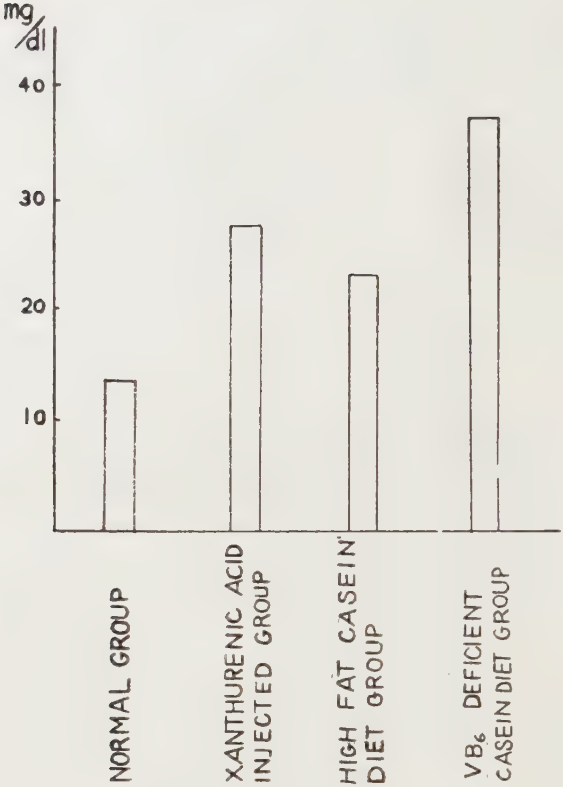

Fig. 5 (The mean value 5 rats)

phate was determined. The results are illustrated in Fig. 6.

As will be seen in the figure blood inorganic phosphate in the healthy control animals decreased. This means that phosphorylation of sugar went on smoothly. But this was not the case with the animals rendered diabetic by the above procedures. Here the decrease of inorganic phosphate did not occur notwithstanding the increase of blood sugar.

D) Some experiments on diabetic patients were carried out as follows: The previously untreated patient of fairly heavy sickness were injected with 50 ml. of 50 per cent glucose solution. In the case of healthy persons such a treatment caused a distinct lowering of blood inorganic phosphate but in the case of diabetic patient rather an increase (Fig. 7).

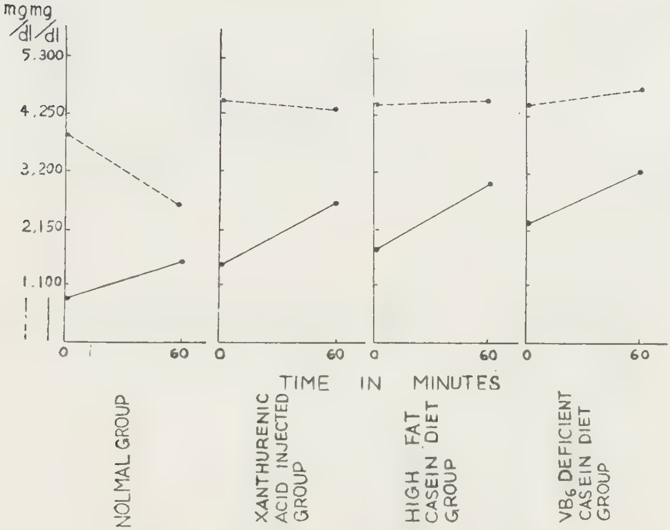

Fig. 6 (The mean value of 5 rats)
- - - - Idorganic phosphate in blood
——— Blood sugar

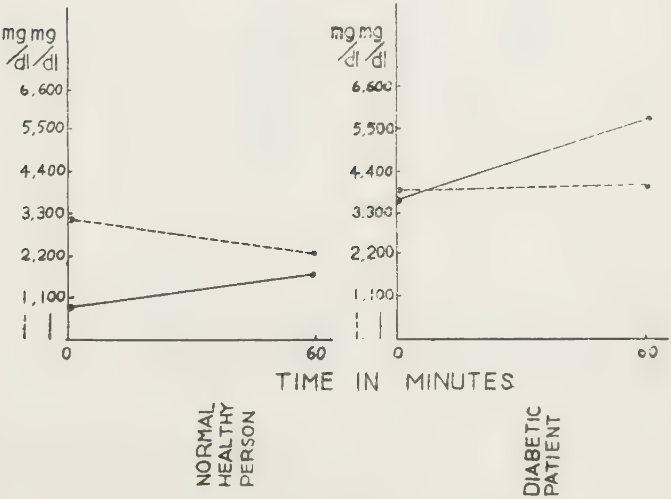

Fig. 7 (The mean value of 2 patients)
——— Blood sugar
- - - - Inorganic phosphate in blood

E) Effect of insulin: Into the rat xanthurenic acid and insulin were applied at the same time, and various determinations as mentioned above were carried out. The results are illustrated in Figs. 8 and 9.

It is clearly shown that insulin suppressed the effects of xanthurenic acid. Blood inorganic phosphate was almost normal and phosphocreatine rather slightly increased, indicating that the phosphorylation recovered to satisfactory.

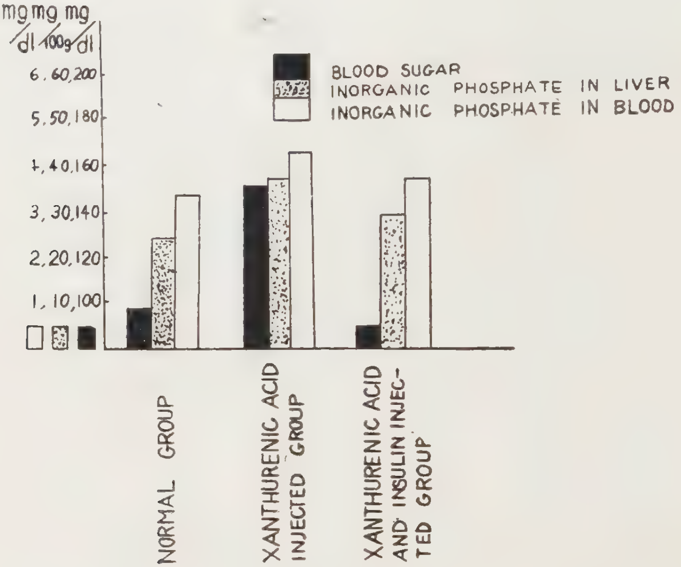

Fig. 8 (The mean value of 5 rats)

DISCUSSION

The above experimental results shows the inhibition of phosphorylation of sugar in the xanthurenic acid diabetes in rat which has been discovered by Kotake and Inada.

Last year Weitzel *et al.* (*15*) confirmed on rabbit the author's findings. Namely he observed that by feeding rabbits on high fat and tryptophan diets xanthurenic acid output in urine increased and at last hyperglycemia and glucosuria were caused to result. The author's finding that xanthurenic acid affords a hinderance in phosphorylating process of sugar in animal body gives an additional evidence to its role

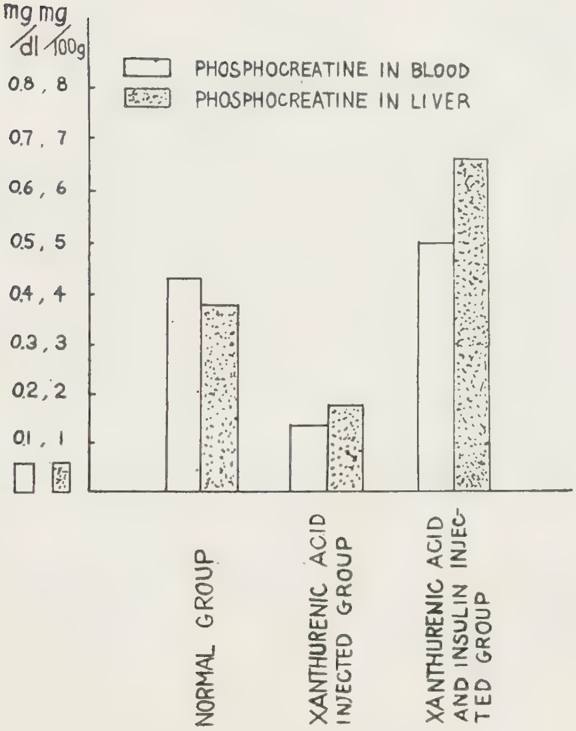

FIG. 9 (The mean value of 5 rats)

as diabetogenic factor.

SUMMARY

1. In rat, when diabetic symptoms exists caused by xanthurenic acid inorganic phosphate in blood and liver increased and phosphocreatine decreased.

2. In such conditions of rat the application of glucose caused no decrease of blood inorganic phosphate just like in the case of diabetic patients.

3. Insulin suppressed the action of xanthurenic acid.

4. In xanthurenic acid diabetes the excretion of total creatinine in urine increased.

We wish to thank heartly to Prof. Yashiro Kotake, for his advice and revision and also heartly thank to Prof. Keizo Kodama for his revision.

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EFFECT OF TRYPTOPHAN AND ANTHRANILIC ACID UPON ADRENALECTOMIZED RAT

I. THE PROLONGATION OF LIFE OF ADRENALECTOMIZED RAT BY THE ORAL APPLICATION OF TRYPTOPHAN AND ITS METABOLITE ANTHRANILIC ACID

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(Received for publication, May 26, 1955)

It is a well known fact that double adrenalectomized animals succumb soon, unless cortical hormones are not provided (*1*) to them.

In rats the death usually comes within 4–12 days after the adrenalectomy, and the survival days are somewhat influenced by animal spesies, sex, feeds and body weight.

This fact was confirmed by the authors on rats fed on the casein diet. As to the medicaments which are effective to prolong the life duration of the operated animals sodium chloride (*2*) and pantothenic acid (*3*) are reported. But the effects of these are almost negligible. In this line the author found out that tryptophan or its metabolite anthranilic acid exerted an influence inducing prolongation of survival duration of the adrenalectomized rats to several extents, the details of which being reported here. The fact seems to give an additional evidence for the postulation of Y. Kotake senior (*4*) that anthranilic acid or 5-hydroxy-anthranilic acid is an important biocatalisator.

Moreover, those metabolites of tryptophan are assumed to hold an interesting physiological relation to the function of suprarenal gland.

EXPERIMENTALS

White rats of various body weight were fed on diet composed of casein 22 per cent agar agar 3 per cent, butter 10 per cent, starch 52 per cent, McCollum salt mixture 6 per cent, yeast 2 per cent and sugar 5 per cent. When the animals showed normal growth on the above diet for 2 weeks, both suprarenal glands were extirpated from back side under ether anesthesy.

The application of tryptophan was done by oral administrating of watery solution or by mixing it into the diet at the intervals given in the table.

Then the survival time was observed and compared with those of control animals.

In all examples the perfect adrenalectomy was confirmed. The rsults are summarised in Table I.

TABLE I

Experimental Rats

(Tryptophan was administered)

Albino rat No.	Body weight	The day of oral administration of 100 mg. tryptophan after the operation conducted	Number of days of survival	Decrease in body weight at time of death
1	290 g.	4th, 11th, 21st, 30th	43	90 g.
2	260	3rd, 8th, 21st	37	80
3	260	2nd, 5th, 9th, 21st	21	30
4	280	2nd, 5th, 9th, 21st	26	60
5	220	2nd, 4th, 11th, 13th 16th, 20th	30	20
6	140	Fed every day on a diet mixed with 15mg of tryptophan	20	0
7	180	7th, 13th, ditto	33	20

Control Rats

(No tryptophan was administered)

Albino rats No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
8	200 g.	4	10 g.
9	260	10	40
10	220	5	15
11	230	9	20
12	180	9	20
13	160	8	10
14	150	8	20

It is noticiable that tryptophan was fairly effective in prolongation of surviving time.

But since it seems more reasonable that anthranilic acid, a metabolite of tryptophan which is assumed to be biocatalyser, is rather essential agency than trypto-

TABLE II

Experimental Rats

(The daily diet was mixed with 2.5 mg of anthranilic acid.)

Albino rat No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
15	140 g.	27	30 g.
16	180	25	20
17	180	20	20

Control Rats

(No anthranilic acid was administered.)

Albino rat No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
18	190 g.	9	45 g.
19	150	8	10
20	180	6	20
21	180	5	40

TABLE III

Experimental Rats

(The daily diet was mixed with 1 mg. of nicotinic acid.)

Albino rat No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
22	180 g.	6	40 g.
23	160	5	20
24	140	5	0
25	160	10	20

Control Rats

(No nicotinic acid was administered.)

Albino rat No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
26	180 g.	9	45 g.
27	180	10	40
28	150	4	15

phan itself, the authors applied it in the same manner as described in the above experiment.

At the same time the effect of nicotinic acid was examined for the sake of comparison.

The above findings clearly show that anthranilic acid has an activity to prolong surviving time, while this is not the case with nicotinic acid. As it is recognized that anthranilic acid passes into 5-hydroxy-anthranilic acid in animal body, perhaps the acid may be an intimate substance which is effective in the action under consideration.

The experiment with 5-hydroxy-anthranilic acid is going on in this laboratory so that in near future the result will be reported.

In the control of the experiments some test on the effects of vitamin C and L-methionine were carried out. The results were all negative as shown in Tables IV and V.

TABLE IV

(The daily diet was mixed with 1 mg. of vitamin C.)

Albino rat No.	Body weight	Number of days of survival	Decrease in body weight at the time of death
29	150 g.	8	30 g.
30	150	7	30
31	150	11	20

TABLE V

(The daily diet was mixed with 20 mg. of L-methionine.)

Albino rat No.	Body weight	Nnmber of days of survival	Decrease in body weight at the time of death
32	180 g.	6	10 g.
33	160	7	30
34	140	11	20

Following the finding as above mentioned that tryptophan and anthranilic acid have the effect of lengthening survival time of adrenalectomized rats it is interesting and important to elucidate the behavior of liver glycogen of adrenalectomized rats under the influence of tryptophan or anthranilic acid. The determination of glycogen was carried out by Kurokawa's method (*5*). The results are indicated in Table VI. (1)—(4)

The data demonstrate that tryptophan and anthranilic acid have a definite influence on holding glycogen content to normal level.

TABLE VI

(The effect of anthranilic acid on liver glycogen
in adrenalectomized rat)

(1) 2.5 mg. of anthranilic acid was daily administered orally after the operation. The glycogen amount was examined in each case on the 6th day.

Albino rat No.	Liver glycogen amount
	g. per cent
35	1.623
36	1.471
37	1.120
The mean value	1.405

(2) 15 mg. of tryptophan was daily administered orally after the operation. The glycogen amount was examined in each case on the 3rd day.

Albino rat No.	Liver glycogen amount
	g. per cent
38	1.447
39	1.473
40	1.698
The mean value	1.539

(3) The glycogen amount of normally healthy rats was examined.

Albino rat No.	Liver glycogen amount
	g. per cent
41	2.000
42	1.744
43	1.585
44	1.570
45	2.050
The mean value	1.789

(4) The glycogen amount was examined in each case on the 3rd day after the operation. This group of albino rats were administered neither with tryptophan nor with anthranilic acid.

Albino rat No.	Liver glycogen amount
	g. per cent
46	0.791
47	1.041
48	0.490
49	0.683
50	0.515
The mean value	0.699

The above data demonstrate the tryptoqhan and anthranilic acid have deflnite influence on holding glycogen content to normal level.

SUMMARY

1. Tryptophan and anthranilic acid are able to keep survival time of adrenalectomized rat several times longer than that of the control animal.

2. The liver glycogen of the operated animal is usually decreased soon, but under the influence of tryptophan and anthranilic acid it is kept well to almost normal level.

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METABOLISM OF TRYPTOPHAN AND TYROSINE IN THE THYROIDECTOMIZED RABBIT

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Kynurenine was discovered by Matsuoka and Yoshimatsu (*1*) in the urine of tryptophan injected rabbits and was investigated and named by Kotake and Iwao (*2*). Iwao repeatedly attempted to isolate kynurenine by injecting tryptophan into normal rabbits without success, until he used a polished rice diet. Lepkovsky and Nielson (*3*) have isolated xanthurenic acid in the urine of tryptophan administrated rats by pyridoxine deficiency, and Kotake, Jr. and Inada (*4*) by butyric acid injection. Recently in our laboratory different conditions (administration of 6-hydroxythiamine, cystine, sodium azide, semicarbazide or acetoacetic acid) were found to decrease kynurenine transaminase in the liver. These facts suggest that the metabolism of tryptophan can be easily disturbed and kynurenine may be excreted in theurine and the question was brought up whether Matsuoka and Yoshimatsu had used the normal rabbits. According to a recent personal communication of Yoshimatsu, the rabbits used in their study may have been thyroidectomized. At that time (1924) the present formula of thyroxine was not yet published and the indole derivative proposed by Kendall was widely accepted. Matsuoka and Yoshimatsu (*5*) had reported in Japanese their studies on the relationship between tryptophan metabolism and the thyroid gland by means of estimation of kynurenic acid in the urine of thyroidectomized rabbits after injection of tryptophan. Consequently their new substance—kynurenine sulfate—must have been found in the urine of thyroidectomized animals.

To confirm this, the following experiments were carried out on thyroidectomized rabbits. Taking this opportunity the metabolism of tyrosine was also studied.

EXPERIMENTAL

Materials

L-*Tryptophan and* L-*Tyrosine*—Preparations of the Kawasaki Pharmaceutical Ind.

DL-*Kynurenine Dihydrochloride*—Furnished by Prof. Sakan. *p*-Hydroxyphenylpyruvate—Synthesized by Kotake's Method (*6*).

α-Ketoglutaric acid, Pyridoxal and ATP—Preparatinos of Wako Pharmaceutical Ind. Ltd.

Enzyme Solution—The rabbits were thyroidectomized without anaesthesia and sacrificed by bleeding 2 to 5 weeks after operation. The liver was ground with equal weights of sea sand and extracted with two (for tryptophan) or three (for kynurenine and tyrosine) times 0.9 per cent KCl solution in the refrigerator for an hour. The extract was centrifuged at 3500 r.p.m. for 15 minutes. The supernatant was used as the enzyme solution. As the control experiment, the rabbits received a sham operation without extirpation of the thyroid gland and were treated afterwards in the same manner as described.

Methods

Enzyme Reaction—L-Tryptophan, DL-kynurenine dihydrochloride, α-ketoglutarate, pyridoxal and ATP were dissolved with $M/25$ phosphate buffer of pH 7.0 at the rate of 1 mg./1 ml., 2 mg./0.5 ml., 2.2 mg./0.2 ml., 200 γ/0.2 ml. and 500 γ/0.5 ml. respectively and adjusted to pH 7.0 with NaOH. The reaction was carried out as floows :

For tryptophan decomposition

Enzyme solution	1.0 ml.
L-Tryptophan solution	1.0 ml.
1 *M* Phosphate buffer (pH 7.2)	0.3 ml.

The mixture was made up to 3.0 ml. by addition of distilled water (pH 7.2) and incubated at 37.5° for 3 hours.

For kynurenine transamination

Enzyme solution	0.1 ml.
DL-Kynurenine-$(HCl)_2$ solution (2 mg.)	0.5 ml.
1 *M* Phosphate buffer (pH 8.2)	0.3 ml.

The mixture was made up to 3.0 ml. by addition of distilled water (pH 8.0) and incubated at 37.5° for 3.5 hours.

Determination of Tryptophan, Kynurenine and Anthranilic Acid—After the reaction, 2.0 ml. of 20 per cent trichloroacetic acid and 1.0 ml. of distilled water were added and filtered. 1.0 ml. of the filtrate was used for the determination. Three substances were determined by the method of Mason and Berg (*7*) modified by Kawamata *et al.* (*8*). The modified method is outlined in the following table.

TABLE I

Compositions of Reaction Mixtures

	(I)	(II)	(III)
Test solution	1.0 ml.	1.0 ml.	1.0 ml.
HCl	0.2 *N* 1.0 ml.	0.2 *N* 1.0 ml.	2.0 *N* 1.0 ml.
$NaNO_2$	0.1% 1.0 ml.	0.1% 1.0 ml.	1.0% 1.0 ml.
Temperature	Room temp.	Room temp.	30°
Reaction time	3 minutes	3 minutes	30 minutes
4% ammonium sulfamate	1.0 ml.	1.0 ml.	4.0 ml.
Temperature	37°	Room temp.	30°
Reaction time	1 hour	3 minutes	10 minutes
Distilled water	5.0 ml.	5.0 ml.	——
0.2% 1-(β-Diethylamino-ethylamino)-naphtalene	1.0 ml.	1.0 ml.	3.0 ml.
Temperature	30°	30°	30°
Reaction time	1 hour	1 hour	30 minutes
Total volume	10.0 ml.	10.0 ml.	10.0 ml.

The violet-red coloured solutions were measured by photometrical determination using a filter S_{55} and a 10 mm.-cuvett. Tryptophan was positive at III only, kynurenine at II and III, and anthranilic acid at all three. The values of the tree substances were easily calculated. Kynurenic acid, 3-hydroxykynurenine, xanthurenic acid and 3-hydroxyanthranilic acid were negative. 5-Hydroxyanthranilic acid gave a violet-blue colour.

Isolation of Kynurenine Sulfate—The original method of Matsuoka and Yoshimatsu was used as described later, although new method for isolating kynurenine sulfate has been reported by Kotake (Sen.) and Shirai (*9*).

Determination of Tyrosine—To the reaction solution, 0.5 ml. of distilled water and 2.0 ml. of 20 per cent trichloroacetic acid were added and filtered. 2.0 ml. of the filtrate were used for the colourimetrical determination by the method of Sidney and Cooper (*10*).

RESULTS

1. Formation of Kynurenine and Anthranilic Acid—The formation of kynurenine and anthranilic acid from tryptophan by the liver extract of the thyroidectomized rabbit 5 weeks after extirpation was far less than that of the Sham operation as shown in Table II.

2. Kynurenine Transminase—While the activity of kynurenine transaminase of liver remained almost unchanged for two weeks after thyroid-

TABLE II

Formation of Kynurenine and Anthranilic Acid from Tryptophan

Experiments were carried out as above described.

	Tryptophan decreased	Kynurenine formed	Anthranilic Acid formed
	γ	γ	γ
Thyroidectomized	183	87	12
Sham operation	256	159	23

ectomy, it decreased markedly after four weeks. By the addition of α-ketoglutarate, pyridoxal and ATP, either singly or combined, it was restored considerably, but was highest with addition of all the three. Anthranilic acid formation from kynurenine by the liver extract of thyroidectomized rabbits was also lowered, but the addition of the above three had no effect (Table III).

TABLE III

Activity of Kynurenine Transaminase of Liver Extract of Thyroidectomized Rabbits

2 mg. of DL-kynurenine dihydrochloride was used. Experiments were carried out as above described.

	Addition			Kynurenine decreased		Anthranilic Acid formed	
After operation	α-Ketoglutarate (2.2 mg.)	Pyridoxal (200 γ)	ATP (500 γ)	Test	Sham	Test	Sham
				γ	γ	γ	γ
2 weeks	−	−	−	124	120		
	+	−	−	276	231		
	−	+	+	125	120		
	+	+	+	305	353		
4 weeks	−	−	−	0	125		
	+	−	−	100	347		
	−	+	+	0	125		
	+	+	+	320	379		
5 weeks	−	−	−	55	186	59	120
	+	−	−	221	443	41	88
	−	+	+	59	187	57	111
	+	+	−	235	448	51	82
	+	+	+	315	495	54	54

3. *Isolation of Kynurenine Sulfate in the Urine of Thyroidectomized Rabbit*—Three g. of tryptophan was finely ground and suspended in 20 ml. of water and injected subcutaneously in a rabbit 2.5 kg. in body weight, 4 weeks after thyroidectomy. The animal became weaker after 16 hours and died within 36 hours. 120 ml. of urine was excreted and 90 ml. left in the bladder. The total urine was evaporated on the waterbath to a syrup and extracted with anhydrous alcohol exhaustively. The alcohol extract was evaporated again to syrup and dissolved with minimal water. The aqueous solution was acidified with sulfuric acid against congo red. Kynurenic acid precipitated promptly. The filtrate separated from the kynurenic acid was kept in the refrigerator over night, and large white needle crystals of kynurenine sulfate appeared. Kynurenic acid was recrystallized with ammonia water and kynurenine was washed with alcohol. The yield of the former was 0.9 g. and that of the latter was 0.5 g. Kynurenine sulfate was recrystallized and identified by means of paper chromatography. Only a single spot of Rf 0.43 was recognized by fluorescence, ninhydrine and Ehrlich's aldehyde reagent.

4. *Transamination of Tyrosine*—As shown in Table IV, transamination of tyrosine was almost lost by the thyroidectomy and completely regained by the addition of α-ketoglutarate, pyridoxal and ATP. Even

TABLE IV

Transamination of Tyrosine by the Liver Extract of Thyroidectomized Rabbit 5 Weeks after Extirpation

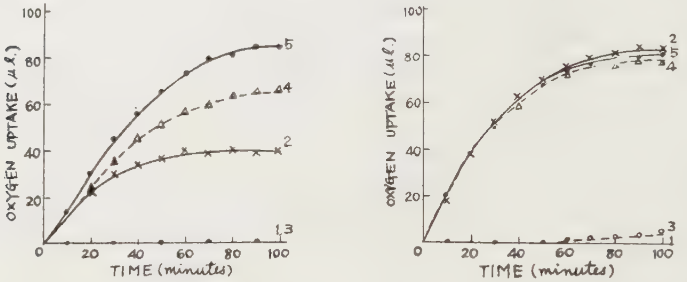
Enzyme 1 ml., 1 M phosphate buffer (pH 7.2) 0.3 ml., tyrosine 2 μM/0.5 ml., α-ketoglutarate 2.2 mg./0.2 ml., pyridoxal 200γ/0.2 ml., and ATP 500 γ/0.5 ml. All were dissolved with M/25 phosphate buffer (pH 7.0) and the mixture was adjusted to pH 7.2 with NaOH and incubated at 37.5° for 100 minutes.

Addition	Thyroidectomized		Sham operation	
	Amount of tyrosine decomposed		Amount of tyrosine decomposed	
	μM	%	μM	%
None	0	0	0.05	2.5
α-Ketoglutarate	1.2	63	2.0	100
Pyridoxal+ATP	0	0	0.08	6.0
α-Ketoglutarate+pyridoxal	2.0	100	2.0	100
α-Ketoglutarate+pyridoxal +ATP	2.0	100	2.0	100

by the simple addition of α-ketoglutarate, it was restored 63 per cent.

5. *Oxygen Uptake by Decomposition of Tyrosine and p-Hydroxyphenylpyruvate*—Both in the test and in the control experiment of tyrosine decomposition, oxygen uptake was not shown without a supplement. In both experiments, pyridoxal and ATP had no effect but by a single addition of α-ketoglutarate, oxygen uptake in the control reached almost to maximum and in the test experiment to one-half (Figs. 1 and 2). In the test experiment, the addition of all three, α-ketoglutarate, pyridoxal and ATP, was shown to give maximal oxygen uptake as high as the control (Fig. 1).

FIG. 1 FIG. 2



1 : none, 2 : α-ketoglutarate, 3 : pyridoxal+ATP 4 : α-ketoglutarate +pyridoxal, 5 : all three.

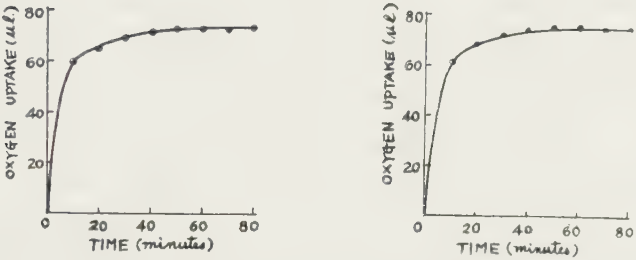
FIGS. 1 and 2. Oxygen uptake by the decomposition of tyrosine 5 weeks after extirpation.

Enzyme solution 1 ml., tyrosine 2 μM, pH 7.2, 37.5°, 100 minutes. The others are the same as described.

In contrast with decomposition of tyrosine, oxygen uptake in p-hydroxyphenylpyruvate decomposition in the test as well as in the control experiment was shown to reach a maximum without addition (Figs. 3 and 4). This can be interpreted to mean, that for the decomposition below p-hydroxyphenylpyruvate, the above three substances were not required.

As the first step in tyrosine decomposition is transamination without oxygen uptake, oxygen is used for the decomposition below p-hydroxyphenylpyruvate. Consequently the minimal uptake of oxygen by the decomposition of tyrosine without addition means minimal transamination.

Fig. 3 Fig. 4



Figs. 3 and 4. Oxygen uptake by the decomposition of *p*-hydroxyphenylpyruvate 5 weeks after extirpation.

p-Hydroxyphenylpyruvate 2 μM. The others are same as in tyrosine decomposition described.

From the *Results 4*) and *5*) tyrosine transamination was found to be markedly disturbed by thyroidectomy but oxidation below *p*-hydroxyphenylpyruvate is left intact.

DISCUSSION

Kynurenine sulfate was isolated by Matsuoka and Yoshimatsu very easily but Iwao was unsuccessful. Since the condition of the rabbits used was not reported, Iwao had used normal animal at first. As described above we have isolated kynurenine sulfate as easily as Matsuoka and Yoshimatsu using thyroidectomized rabbit. Putting together various circumstances as described in the introduction it can be concluded, that Matsuoka and Yoshimatsu must have used thyroidectomized rabbits.

The activity of kynurenine transaminase of the liver extract was markedly decreased by thyroidectomy (Table II), by polished rice diet (*11*), by administration of 6-hydroxythiamine (*12*), cystine (*13*), semicarbazide (*14*), natrium azide (*15*) and acetoacetic acid. Lepkovsky *et al.* (*3*) have isolated xanthurenic acid in the urine of pyridoxine deficient rats after tryptophan administration, and Kotake, Jr. *et al.* (*4*) by administration of butyrate. In the urine of severe human tuberculosis, kynurenine (*16*), 3-hydroxykynurenine (*17*) and xanthurenic acid (*18*) were detected and the latter was also found in diabetic urine (*19*). Consequently kynurenine was accumulated by various conditions and partially eliminated in the urine and was partially decomposed through the pathway of nucleus oxidation.

Not only knyurenine transamination but the formation of kynurenine from tryptophan and that of anthranilic acid from kynurenine were also disturbed by thyroidectomy (Tables II and III). Deffering from kynurenine transamination, the anthranilic acid formation was not improved by the addition of α-ketoglutarate, pyridoxal and ATP (Table III).

Transamination of tyrosine by the liver extracts of the control and thyroidectomized animals were found to be very low and when α-ketoglutarate was added, the former was increased by 100 per cent and the latter by 63 per cent. When α-ketoglutarate, pyridoxal and ATP were added, transamination of tyrosine by the thyroidectomized liver extract was increased by 100 per cent (Table IV).

Transamination without oxygen uptake takes place when tyrosine is converted to *p*-hydroxyphenylpyruvate and oxygen uptake occurring in tyrosine decomposition is used for the oxidation below *p*-hydroxyphenylpyruvate. As shown in Table IV transamination of tyrosine by liver extracts was nearly zero without addition, oxygen uptake did not occur by tyrosine decomposition without addition (Figs. 1 and 2). In coincidence with transamination (Table IV), oxygen uptake was shown by tyrosine decomposition (Figs. 1 and 2). On the other hand in the decomposition of *p*-hydroxyphenylpyruvate, oxygen uptake was found in the test as well as in the control experiment without addition (Figs. 3 and 4). That is to say the thyroid galnd does not seem to affect the oxidation below *p*-hydroxyphenylpyruvate much.

SUMMARY

1. Kynurenine sulfate was isolated by the simple method of Matsuoka and Yoshimatsu from the urine of thyroidectomized rabbit administrated tryptophan four weeks after extirpation. It was suggested that their rabbits must have been thyroidectomized.

2. Four weeks after the extirpation, kynurenine transamination of the liver extract was decreased and was improved by the addition of α-ketoglutarate, pyridoxal and ATP. Within two weeks it remained intact.

3. The formation of kynurenine from tryptophan and that of anthranilic acid from kynurenine by liver extract were also disturbed by the thyroidectomy and the latter was not found to be improved by the addition of the above three substances.

4. Tyrosine transamination was decreased by the liver extract of the thyroidectomized animals and was regained completely by addition of the above three substances. By thyroidectomy oxygen uptake during the decomposition of *p*-hydroxyphenylpyruvate was not found to be disturbed. The relationship between thyroid gland and tyrosine decomposition was discussed.

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STUDIES ON THE FORMATION OF INDOLE AND PHENOL FROM TRYPTOPHAN AND TYROSINE RESPECTIVELY FROM THE STANDPOINT OF THE NUCLEUS

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Ichihara and Iwakura (*1*) have reported that DL-indole lactic acid replaces tryptophan in the nutrition of rats, Saito (*2*) has reported the formation of indole from DL-indole lactate and indole pyruvate by *E. coli*. These facts suggest that they were converted to tryptophan in the bacterial cells and from this, indole was formed. From this and other results, Ichihara (*3*) came to the conclusion that indole might be formed from tryptophan only. Baker and Happold (*4*) have stated from studies of over 20 indole derivatives, that for the formation of indole from tryptophan an intact side chain is required. Later it was proven that for the formation of phenol from tyrosine (*5*) and methylmercaptane from methionine (*6*) the same holds true.

On the other hand, it has not been shown that benzene and imidazole are formed from phenylalanine and histidine respectively which have the same side chain as tryptophan and tyrosine. Consequently, the nucleus of tryptophan and tyrosine and the biological splitting of the nucleus cannot be considered separately.

According to Yoritaka (*7*), indole which is not substituted at the α- or β-position gives the Ehrlich's aldehyde reaction. The substituted products of tryptophan could therefore easily be detected, and from *o*- and *m*-tyrosine the same phenol as from the *p*-form had to be formed, if they were to be produced.

EXPERIMENTAL

Materials

pr-a-Methyltryptophan and o- and m-Tyrosine—*pr*-Methyltryptophan and *o*- and *m*-

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This work is supported by research grant from the Department of Education, and was reported at the meeting of Osaka Medical Association Oct. 1950 and Oct. 1951.

tyrosine were synthesized by the modified methods of Matsui *et al.* (*8*). The respective aldehydes were condensed with hydantoin in piperidine. The melting points of *pr-α*-methyltryptophan, *o*- and *m*-tyrosine were 255–256°, 251–255°, and 283–289°, respectively.

pr-N-Methyltryptophan—Synthesized by the method of Wieland *et al.* (*9*), m.p. 285°.

pr-α-Hydroxytryptophan—Furnished by Prof. Sakan (*10*)

Methods

Stranis—*E. coli phenologenes* and *E. coli indologenes* were isolated from feces and cultivated on pepton agar plates. The cells were harvested and washed until the wash water no longer gave the phenol and indole reactions. As agar disturbs the experiment, it must be removed completely. The strain was inoculated in 5.0 ml. of Zipfel's synthetic medium of the following composition: 200 mg. of the substance to be tested : NaCl, 5.0 g. ; KH_2PO_4, 2.0 g. ; $(NH_4)_2$-CO_3, 1.0 g. ; $MgSO_4$, 0.1 g. ; tap water, 1 liter.

Determination of Indole—Indole was determined by the method of Goto (*11*). An aliquot of reaction liquid was extracted by petroleum ether and after adding Ehrlich's reagent, the petroleum layer was photometrically determined.

Determination of Phenol—Phenol was determined by the method of Kakihara and Ichihara (*5*). An aliquot of reaction liquid was extracted by petroleum ether and after adding diazobenzolesulfonic acid reagent and Na_2CO_3 solution, the petroleum layer was photometrically determined, using a S_{47} filter and a 10 mm.-cuvett.

TABLE I

Formation of Substituted Indole from Substituted Tryptophan Incubated for 16 *Hours*

Tested substance	Tryptophan-(Control) (1 mg.)	pr-α-Methyl-tryptophan (1 mg.)	pr-α-Hydroxy tryptophan (1 mg.)	pr-*N*-Merhyl-tryptophan (1 mg.)
Indole formed γ	32.4	0	0	0

TABLE IV

Formation of Phenol from o- and m-Tyrosine Incubated for 16 *Hours*

Tested substance	*p*-Tyrosine (Control) (1 mg.)	*o*-Tyrosine (1 mg.)	*m*-Tyrosine (1 mg.)
Phenol formed γ	16	0	0

DISCUSSION

It has already been reported by Baker *et al.* (*12*) that substituted indole could not be formed from pr-*N*-methyltryptophan. From the results, it was confirmed further that substituted indoles could not be formed from pr-α-methyl-, pr-α-hydroxy- and pr-*N*-methyltryptophan. When Majima (*13*) obtained D-pr-α-methyltryptophan from the DL-form by means of decomposition of L-moiety by *E. coli*, α-methyl indole was reported to be formed. As it was a macro-scale experiment, this result may be attributed to the impurity of material used.

According to personal communications of Happold the corresponding indole was produced by *E. coli* from 4-, 5, 6- and 7-substituted tryptophans. 5-Methylindole was reported to be formed from 5-methyltryptophan by *E. coli* (*14*) in the presence of L-tryptophan.

It is clear consequently, that for the indole formation two hydrogen atoms (1 and 2) of pyrrole of tryptophan must not be substituted. The benzene moiety is indifferent. Baker *et al.* (*12*) made note of hydrogens of 1 of indole and β of the side chain but overlooked that of the 2 position.

Phenol was also formed only from *p*-tyrosine and from neither *o*- nor *m*-forms.

The authors considered the formation of kynurenine and homogentisic acid. The former was produced from tryptophan but not from pr-α-hydroxytryptophan (*15*, *16*, *17*) and the latter from *p*-tyrosine (and phenylalanine) but not from the *m*-form (*18*). The mechanisms of formation of indole and kynurenine as well as that of phenol and homogentisic acid are of course not similar. However it can be said, that for their formation, the hydrogen atoms of pyrrole of tryptophan and *p*-hydroxybenzene of tyrosine must not be substituted. Substituted kynurenines on the other hand may be expected to be produced from bz-substituted tryptophan as indole formation.

SUMMARY

1. Substituted indole could not be formed from pr-α-methyl-, pr-α-hydroxy- and pr-*N*-methyltryptophan by *E. coli* indologenes.

2. From *o*- and *m*-tyrosine, phenol could not be formed by *E. coli phenologenes.*

3. The relationship between the formations of indole, kynurenine, phenol and homogentisic acid and the nucleus of their mother amino

acids was discussed, and the formation of bz-substituted kynurenine from bz-substituted tryptophan was anticipated.

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THE EFFECT OF LIGHT ON THE METABOLISM OF LACTIC ACID BY RHODOPSEUDOMONAS PALUSTRIS. I

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It has already been observed by many workers that the rate of respiratory uptake of oxygen by photosynthesizing purple bacteria is more or less decreased by the effect of illumination. Nakamura (*1*) who worked once in our laboratory, has interpreted the phenomenon as due to the partial cancelling off of the respiratory O_2-uptake by photosynthetic O_2-evolution which he believed to occur also in the bacterial photosynthesis. The possibility of production of molecular oxygen by the bacteria in the light has since been examined by many workers (see (*2*)), but always with negative results. The *coup de grâce* to Nakamura's claim was recently given by Johnston and Brown (*3*) who showed, by following the fate of isotopically marked oxygen, that the light caused an inhibition of uptake, not a complementary evolution of molecular oxygen. However, the mechanism by which light inhibits the respiratory oxygen uptake by purple bacteria has not yet been elucidated. The present study was undertaken in an attempt to get an insight into the mechanism of the phenomenon in question.

MATERIALS AND METHODS

A strain of *Rhodopseudomonas palustris* (Molisch) Kluyver et vanNiel, a typical non-sulfur purple bacterium was used throughout the experiments. The bacterium was cultured for 48 hours at 25–27° under continuous illumination in a medium consisting of 0.5 per cent " polypeptone " and 0.5 per cent sodium lactate (pH 7.0). The cells were harvested by centrifugation and were washed several times with distilled water (*4*, *5*).

Oxygen uptake was measured at 27° in a Warburg manometer. The main chamber of the manometer vessel contained the bacterial cells (usually 10-30 mg. in dry weight) suspended in phosphate buffer of pH 7.0, to which a solution of the substrate, lactic or pyruvic acid, which previously had been neutralized to pH 7.0 with sodium hydroxyde, was tipped in from the side bulb of the vessel. The center well was provided with 0.5 ml. of 20 per cent slution of potassium hydroxyde to absorb the carbon dioxide evolved. The gas phase was filled with air, unless otherwise stated. All values for oxygen consumption given in the tables and figures were corrected for endogenous respiration.

The illumination of the manometer vessel was furnished by a 100 watt incandescent lamp placed beneath the glass window at the bottom of the thermostat. Adjustment of the light intensity was effected by varying the distance of the lamp from the vessel. In most of the experiments, the incident light intensity was 2,000–5,000 lux which was saturating in the sense that higher intensities brought about no further light-effect on the metabolism.

Lactic and pyruvic acids respectively were colorimetrically determined by the methode of Barker and Summerson (*6*) and of Friedman and Haugen, modified by Shimizu (*7*). 1, 2, 3-C^{14}-labeled lactic acid was prepared by the fermentation (with *Lactobacillus casei*) of homogeneously C^{14}-labeled glucose which was obtained, according to Hassid (*8*), by the acid-hydrolysis of starch photosynthesized from radioactive carbon dioxide by excised leaves of *Aster*. Carboxyl-C^{14}-labeled lactic acid was prepared chemically according to the method of Cremer and Kistiakowsky (*8*). Radioactivity of the samples was measured with the Geiger-Müller counter ; corrections were made both for back-ground counts and for the effect of self-absorption by the sample being counted.

RESULTS

Effect of Light on the Oxygen Uptake and the Breakdown of Lactic Acid—The bacterium, when kept in darkness in the presence of lactic acid, showed an oxygen uptake, the rate of which remained steady for an appreciable length of time until it suddenly fell off with the exhaustion of the substrate in the reaction medium. The steady rate of O_2-uptake was more or less suppressed when the system was illuminated, and on turning off the light it recovered almost immediately to the original level observed in the darkness. One of the typical results obtained on repeated alternation of dark and light is reproduced in Fig. 1 (see also Fig. 6).

It has previously been shown (*4*) that the bacterium used in the present experiment produces a certain amount of molecular hydrogen on illumination. The same phenomenon has also been observed by Gest and Kamen (*9*) in *Rhodospirillum rubrum*. It may, therefore, be conjectured that the apparent decrease of O_2-uptake caused by the light could possibly be due to the photochemical evolution of molecular hydrogen. That this possibility can hardly account for the observed

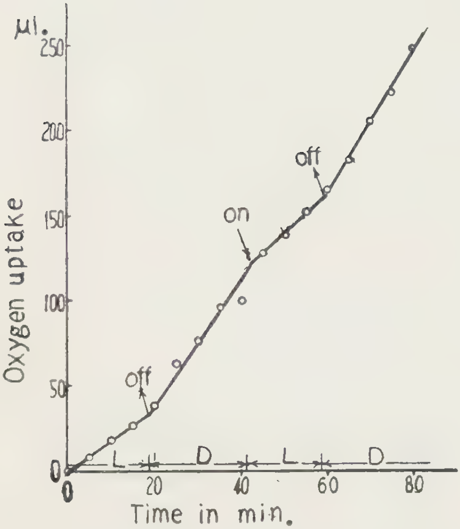

FIG. 1. Effect of light on the oxygen uptake in the presence of lactic acid as substrate. Experimental solution contained per vessel ; 250 μM lactic acid, and 19.6 mg. (dry weight) bacteria. Suspension volume, 5 ml. Ordinate values are corrected for endogeneous respiration.

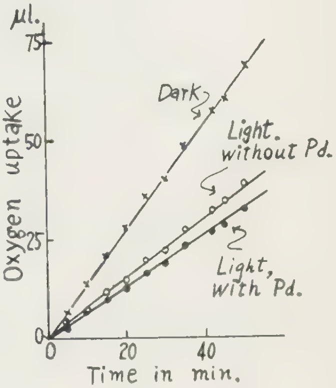

FIG. 2. Effect of the presence of palladium asbestos (in the side bulb) on gas uptake in the light. Lactic acid, 50 μM ; bacteria, 3.6 mg. (dry weight) per vessel. Suspension volume, 5 ml. Crosses : oxygen uptake in the dark (without palladium), solid and open circles : oxygen uptake in the light, with and without palladium, respectively.

phenomenon may be seen from the experimental results presented in Fig. 2 ; the presence of palladium asbestos in the side bulb of the manometer vessel did not bring about a recovery of the respiratory rate suppressed by the effect of light.

The light-effect observed in the above experiments, which was shown to be virtually a decrease of O_2-uptake itself, increased with the intensity of light applied as shown by the curve in Fig. 3. Additional measurements at higher light intensities (not included in the figure) showed that, at light intensities above *ca.* 1,5000 lux, the depressing light-effect became constant ; the rate of O_2-uptake was almost exactly

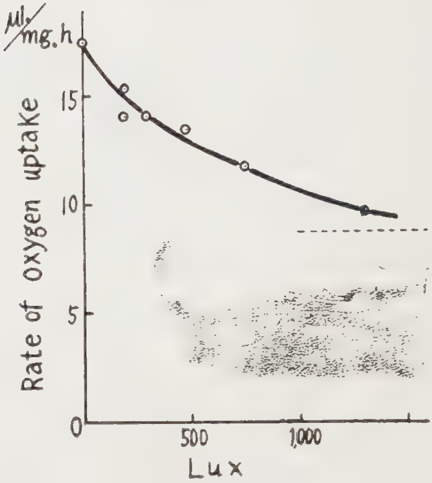

FIG. 3. Rate of the oxygen absorption as a function of light intensity. Lactic acid, 250 μM ; bacteria, 13.2 mg. (dry weight) per vessel. Suspension volume, 5 ml. The broken line indicates the final level of oxygen uptake attained at higher intensities (2000–6000 lux) of illumination.

50 per cent of that observed in the darkness.

By incubating the same amount of bacterial cells in the darkness, on one hand, and in the light of sufficiently high intensity (2,000–5,000 lux), on the other, it was found that, whereas the rate of O_2-uptake was 50 per cent suppressed by the light, the rate of consumption of lactic acid remained the same in both cases. The results presented in Table I and Fig. 4 show that, per mole of lactic acid disappearing, 1 mole of O_2 was absorbed in the dark, while only 1/2 mole of O_2 was taken up under strong illumination. The same fact was confirmed when we measured the total amount of O_2 which had been absorbed at the time of exhaustion of the lactic acid supply. Exhaustion of the substrate was manifest in the sudden decrease of the rate of O_2-uptake to the level of endogenous respiration, at which time, practically no lactic acid was detectable in the reaction medium.

Effect of Sodium Arsenite on the Breakdown of Lactic Acid—Sodium arsenite was found to cause a significant alteration in the process of breakdown of lactic acid by the bacterium. With $10^{-3}M$ arsenite,

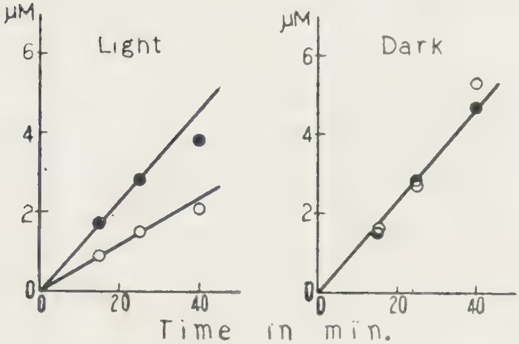

Fig. 4. Oxygen uptake and consumption of lactic acid. Lactic acid, 10 μM ; bacterial cells, 30 mg. (dry weight) per vessel. Suspension vloume, 5 ml. The oxygen uptake was corrected for endogeneous respiration. Open circles, oxygen absorbed ; solid circles, lactic acid consumed.

TABLE I

Effect of Light on the Oxygen Uptake and Consumption of Lactic Acid

Experimental solution contained per vessel ; 10 μM Na lactate and 30 mg. (dry weight) bacteria. Suspension volume, 5 ml.

	Drrk		Light	
Time	Oxygen absorbed	Lactate used	Oxygen absorbed	Lactate used
min.	μM	μM	μM	μM
15	1.6	1.5	0.9	1.7
25	2.6	2.8	1.5	2.8
40	5.3	4.7	2.1	3.6

the rate of oxygen uptake was inhibited about 84 per cent in dark, and about 70 per cent in the light (Table II). Of interest is the fact that, while the rate of O_2-uptake was reduced to 50 per cent by the effect of light in the absence of arsenite, it remained unaffected by the light in the presence of arsenite (Table II). In an experiment similar to that given in Table II, after the cells had absorbed an appreciable amount of O_2, the reaction medium was analysed for lactic acid and for pyruvic acid (which is the most probable primary oxidation product derived from lactate (Table III)). It was revealed by this experiment that 1) pyruvate was accumulated, both in the light and dark, only in the presence of arsenite, and not in its absence and 2), in the presence of

TABLE II

Effect of Arsenite on the Rate of Oxidation of Lactic Acid

Sodium arsenite : $10^{-3}M$ (final concentration) ; lactate : 250 μM ; bacterial cells : 22.4 mg. Suspension volume, 5 ml.

	Without arsenite	With arsenite
Dark	278.0 *μl./hr.*	45.4 *μl./hr.*
Light	142.7	42.3

TABLE III

Formation of Pyruvic Acid from Lactic Acid in the Presence of Arsenite

Bacteria : 22.4 mg. (dry weight) per vessel ; initial amount of lactic acid : 250 μM. Arsenite : $10^{-3}M$. Suspension volume, 5 ml. Quantities of O_2 absorbed and pyruvate formed were measured after 55 minutes' incubation.

		Oxygen absorbed	Pyruvic acid accumlated
With arsenite	Dark	2.1 μM	3.3 μM
	Light	2.2	5.4
Without arsenite	Dark	12.5	0.0
	Light	6.0	0.0

arsenite, and in both light and dark, roughly 1/2 mole of O_2 was consumed per one mole of pyruvic acid produced.

Effects of Light on the Breakdown of Pyruvic Acid—The fact described above makes it appear highly probalble that the effect of arsenite lies in

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* Tsukamoto (*11*), in our laboratory, who worked with the same strain of the bacterium as ours, has found that pyruvic acid is formed from lactate in the absence of arsenite. The discrepancy between our findings may be due to the difference in the way of preparing the suspension of bacterial cells. Cells used in Tsukamoto's experiments had been previously starved by aerating the organism under dim light, for 3 days at 27°, whereas the material for the present studies were the cells freshly harvested and washed several times (10–15 times) on a centrifuge with distilled water or with dilute phosphate buffer.

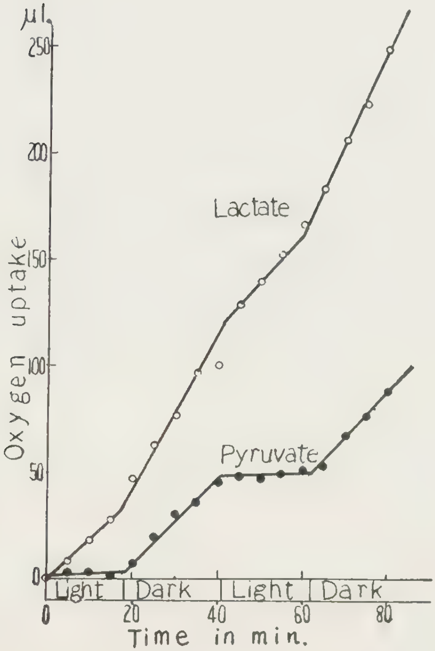


Fig. 5. Oxygen absorption in the light and in the dark. Substrate : lactic acid, 250 μM ; or pyruvic acid, 250 μM ; bacterial cells : 19.6 mg. (dry weight) per vessel. Suspension volume, 5 ml. Intensity of illumination : 4,000 lux. The oxygen absorption was corrected for the endgeneous respiration.

the blocking of the further breakdown of pyruvate which is conceivably formed as the primary oxidation product from lactate. In connection with this assumption it is pertinent to describe our observation regarding the effect of light on the breakdown of pyruvic acid.

In Fig. 5 are reproduced the results of an experiment in which the effect of light and darkness on O_2-uptake in the presence of pyruvate was compared with that observed in the presence of lactate. As is apparent from the curves, the O_2-uptake in the presence of pyruvate was more strongly affected, and indeed completely suppressed by the effect of light. It may also be seen that in the dark the rate of O_2-uptake due to pyruvate oxidation was approximately half of that caused by lactate oxidation.

By incubation of bacteria with pyruvate in the dark under aerobic conditions, it was found that approximately 1/2 mole of oxygen was absorbed per mole of pyruvate consumed (see Tables IV and V). A highly interesting fact was revealed when we investigated the effect of light upon the breakdown of pyruvate (Table IV). Whereas the O_2-uptake was almost completely suppressed by the light, the rate of consumption of pyruvate remained practically unaffected by illumination. This observation points to the fact that in the light pyruvate was broken down non-oxidatively. Further evidence in support of this inference was provided by an experiment, in which the effect of light on pyruvate breakdown was measured under aerobic and under anaerobic conditions (Table VI) ; Pyruvate was consumed in the dark only aerobically but illuminated cells metabolized pyruvate as rapidly under anaerobic as under aerobic conditions.

TABLE IV

Rate of Oxidation of Pyruvic Acid in the Light and Dark

Bacterial cell : 19.6 mg. (dry weight) per vessel ; initial amount of pyruvic acid : 25 μM. Suspension volume, 5 ml. Intensity of illumination : 4,000 lux.

	Consumption of pyruvate	
Time	Light	Dark
30 *min.*	3.3 μM	3.3 μM
60	17.5	13.6
90	21.8	17.4
Average rate	16 μM/*hr.*	12 μM/*hr.*
Rate of oxygen uptake	0.5 μM/*hr.*	5.8 μM/*hr.*

TABLE V

Total Oxygen Absorbed (in the Dark) for Consumption of 4.16 μM. Pyruvic Acid

Pyruvate consumed	Oxygen absorbed	$\frac{\text{Pyruvate}}{\text{Oxygen}}$
4.16 μM	2.41 μM	1.7

Effect of Sodium Arsenite on Pyruvate Breakdown in the Light and in the Dark—The proceeding experiment showed clearly that the fate of pyruvate was strikingly dependent on whether of not the reaction system was illuminate. These two modes of pyruvate breakdown, namely the oxidative one occurring in the dark and non-oxidative one occurring in the light, were found to be differently affected by arsenite. In Fig. 6

TABLE VI

Effect of light on the Consumption of Pyruvic Acid under Aerobic and Anaerobic Conditions

Bacteria : 10 mg. (dry weight) per vessel, initial amount of pyruvic acid : 1 μM ; reaction time : 45 min. ; light intensity : 4,000 lux. Suspension volume, 5 ml.

	Pyruvate consumed	
	In air	In nitrogen
Light	0.86 μM	0.87 μM
Dark	0.82	0.05

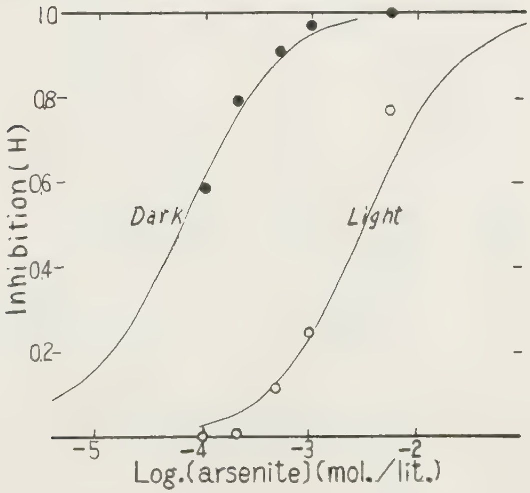


FIG. 6. Effect of arsenite on the consumption of pyruvic acid. Pyruvic acid : 2.5 μM ; bacterial cells : 12.2 mg. (dry weight) per vessel. Suspension volume, 5 ml. Intensity of illumination : 4,000 lux. Duration of the reaction : 60 min.

are reproduced the results of experiments, in which the effect of varying concentrations of arsenite upon the rate of aerobic consumption of pyruvate was investigated in the light and in darkness. In this figure, the ordinate represents the degree of inhibition (H) which was defined as:

$$H=1-\frac{V_g}{V}$$

where V_g and V are the rates of consumption of pyruvate in the presence and absence, respectively, of arsenite. The continuous curves in the figure, which approximately fit the experimental values, are those calculated by

$$H=\frac{[G]}{\phi-[G]}$$

where [G] is the concentration of arsenite added, and ϕ a constant which corresponds to the concentration of the poison causing 50 per cent inhibition. The value of ϕ was $10^{-4.2}$ *M* in the dark, and $10^{-2.6}$ *M* in the light, which means that the dark reaction was about 40 times more sensitive towards arsenite than the light reaction.

Experiments with 1,2,3-C¹⁴-labeled Lactic Acid—In order to trace the details of the metabolism of lactic acid, the breakdown of 1,2,3-C^{14}-labeled lactic acid was investigated. The suspension of bacterial cells was place in the main chamber of a Warburg vessel, whose side bulb was replaced with a tightly fitting rubber stopper, through which a solution of 1,2,3-C^{14}-labeled lactate could be introduced by means of a glass syringe (see Fig. 7). The main opening of the vessel was closed with a glass stopper and the center well was filled with 20 per cent KOH-solution. For each experiment (performed in the light and dark) three reaction-vessels of the same arrangement were run in parallel. After the vessels were brought to the temperature equilibrium (27°), the reaction was started by introducing the substrate from the syringe, and the vessels were continuously shaken thereafter. At 30, 60, and 90 minutes from the time of addition of labeled lactate, the reaction in one vessel was stopped by introducing 0.5 ml. of 20 per cent trichloracetic acid (TCA). The vessel contents were separated into three fractions as follows: after standing for 15 minutes at room temperature, the bac-

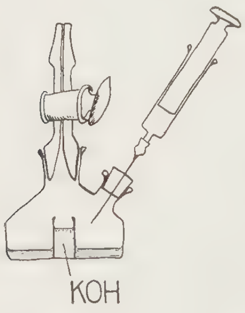


FIG. 7. The vessel used in the experiments with radioactive carbon.

terial cells precipitated with TCA were separated from the solution, by centrifugation, washed several times with distilled water, and dried (*Fraction ii*) ; the supernatant was neutralized and combined with the washings (*Fraction ii*) ; $BaCl_2$ was added to the alkali in the center well and the precipitate was dried (*Fraction iii*). The radioactivity of each fraction was measured. Furthermore, using aliquots of the supernatant, the quantity of lactic acid remaining in the reaction mixture was measured colorimetrically. The results obtained are illustrated in Fig. 8.

As has been stated already, the decrease in the concentration of lactate was not significantly influenced by the effect of illumination. The coincidence of the curve representing the decrease in the radioactivity of the supernate with that for the concentration of lactate indicates that the radioactivity of the supernatant was solely due to the remaining lactate.

The radioactivity both in the TCA-precipitated cells (i) and in the

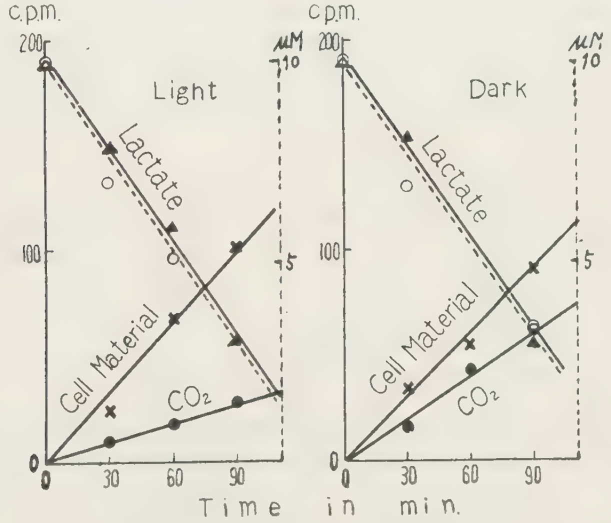


FIG. 8. The fate of radioactive carbon during the oxidation (in light and dark) of uniformly C^{14}-labeled lactate. Substrate : 1,2,3-C^{14}-lactic acid, 10 μM, 190 c.p.m. ; bacterial cells : 19 mg. (dry weight) per vessel. Suspension volume, 5 ml. Ordinate values for c.p.m. are those per vessel shown by lactic acid (open circles), the supernate (solid triangles), the TCA-precipitated cells (corsses), and carbon dioxide (solid circles).

carbon dioxide absorbed by KOH (iii) increased linearly with time. In the course of the reaction lasting 90 minutes about 70 per cent of the added lactic acid disappeared from the reaction mixture. Of that which disappeared in the dark, 43 per cent of the radiocarbon was recovered as carbon dioxide and 67 per cent was found incorporated in bacterial cells; corresponding values in the light were 20 per cent for carbon dioxide and 74 per cent for bacterial cells. It should be noted that the light, which did not affect the consumption of lactated, increased to some extent the incorporation of C^{14} into bacterial cells, and reduced the production CO_2 from lactate to about one half of the corresponding value observed in darkness.

The balance sheets of C^{14}-counts found at the 90 th minute of the experiment are as follows:

In the dark—

Lactic acid consumed:	136 c.p.m. (7.2 μM)
CO_2 evolved:	60 c.p.m.
TCA-precipitated bacterial cells:	92 c.p.m.

Considering the amount of O_2 absorbed in the meantime, the ratio of lactic acid consumed (L), oxygen absorbed, CO_2 evolved, and the carbon assimilated in the TCA-precipitated cells (C) is approximately in the following ratio:

$$L : O_2 : CO_2 : C = 1 : 1 : 1.3 : 2.0$$

In the light—

Lactic acid consumed:	136 c.p.m. (7.2 μM)
CO_2 evolved:	26 c.p.m.
TCA-precipitated bacterial cells:	98 c.p.m.

The ratio in this case is approximately as follows:

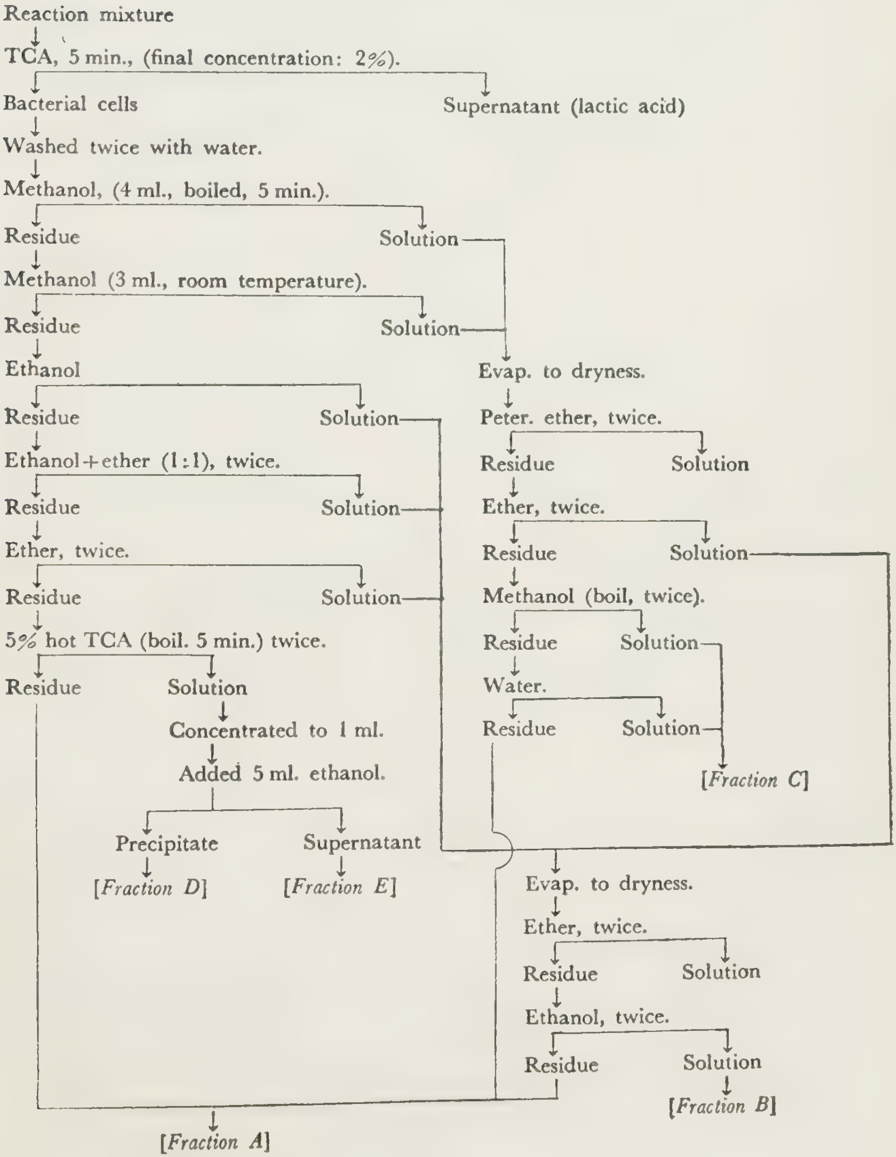
$$L : O_2 : CO_2 : C = 1 : 0.52 : 0.57 : 2.2$$

Experiments comparable to the one described above were performed on a somewhat larger scale, and the TCA-precipitated cells were analysed to determine the distribution of racioactive carbon among the cellular constituents. The methods of fractionation is shown by the flow sheet given in Table VII.

Fraction A given in the flow sheet may be considered to consist mostly of protein, *Fraction B* mainly of lipids, and *Fraction C* probably of carbohydrate, peptides and other substances of low molecular weight. *Fraction D* may contain groups of substances easily hydrolysable by acids, carbohydrates, peptides and phosphate esters inculuding nucleic acids.

TABLE VII

Flow Sheet of Fractionation of TCA-precipitated Bacterial Cells which Had Metabolized Homogeneously C^{14}-lacbeled Lactate



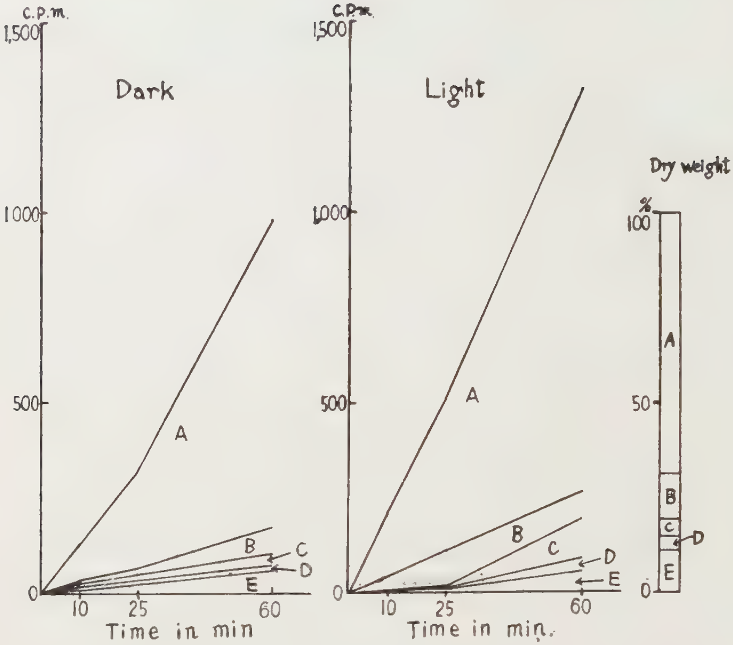


FIG. 9. Assimilation of C^{14} from homogeneously C^{14}-labeled lactate, and its distribution in various fractions in TCA-precipitated bacterial cells. Substrate : 1,2,3-C^{14}-lactic acid, 5 μM, 4,288 c.p.m. ; bacteria : 30.7 mg. (dry weight). c.p.m. : radioactivity of each fraction per vessel. Suspension volume, 5 ml. For explanation of each fraction see text.

Fraction E may be of composition similar to *Fraction D*, but will contain substances of lower molecular weight.

The time course of C^{14}-incorporation in these fractions are shown in Fig. 9. The inset in the figure shows the relative amount (in per cent of dry weight) of each fraction, which remained practically unchanged during the course of experiment of C^{14}-provision. Owing to the scarcity of the material, further analysis for the distribution of C^{14} in each fraction could not be carried out. The fact, however, that the radioactive counts of each fraction increased in roughly in a definite proportion suggests that, as far as the present experiments were concerned, the radioactive carbon atoms were fairly evenly distributed among the various components of the cell material.

TABLE VIII

Distribution of C^{14} in the TCA-precipitated Bacterial Cells and the CO_2 Evolved during the Metabolism of Carboxyl-C^{14}-labeled Lactic Acid

Bacterial cells : 13 mg. (dry weight) per vessel. Initial amount of lactate : 3.6 μM, 705 c.p.m. Suspension volume, 5 ml. Reaction time : 30 min.

	C^{14} incorporated in	
	TCA-precipitated cells	CO_2
Dark	55 c.p.m.	324 c.p.m.
Light	129	138

Experiment with Carboxyl-labeled Lactic Acid—An experiment similar to that described above was performed using carboxyl-C^{14}-labeled lactic acid as substrate. The bacteria were incubated, in both light and dark, for 30 minutes with the substrate, and the carbon dioxide evolved and the TCA-precipitated bacterial cells were separately analysed for their radioactivity. As may be seen from the data given in Table VIII, carboxyl-C^{14} was metabolized in the dark to a larger extent (54 per cent) than in the light (38 per cent). Of the total carboxyl-C^{14} disappearing in the dark, 14 per cent was recovered in the TCA-precipitated cells and 86 per cent wss liberated as carbon dioxide. The corresponding values observed in the light were 48 and 52 per cent, respectively. The ratio of radioactivity in carbon dioxide evolved and incorporated into TCA-precipitated cells was as follows : 1 :0.17 in the dark, and 1 :0.93 in the light.

The fact that the evolution of C^{14}-containing carbon dioxide was lower in the light than in the dark might be construed as being due to the photosynthetic assimilation of a part of the $C^{14}O_2$ which was liberated from the carboxyl group. To investigate whether the existence of a large quantity of non-radioactive CO_2 will affect the utilization of C^{14} in the carboxyl-labeled lactate, the following experiment was carried out. Use was made of the vessel illustrated in Fig. 7, with the modification that the main opening (at the top of the vessel) was also closed with a rubber stopper. In one series of experiments, the gas space of the vessel was filled with air containing 5 per cent (unlabeled) CO_2, the center well being left empty. In another series of experiments, the gas space of the vessel was filled with CO_2-free air, and the center well was provided with 20 per cent KOH solution. After the carboxyl-labeled lactate was introduced (by the use of an injection syringe through the rubber stopper in the side opening) into the bacterial suspension placed in the main chamber, the vessels were shaken at 27°. After certain periods of time, 20 per cent KOH solution was introduced (by the use of syringe through the rubber stopper of main opening) into the center well of the CO_2-containing vessel, and the shaking was continued for 15 minutes to fix the CO_2 by the KOH solution. Measurement of radioactivity fixed in the KOH solution and that in the TCA-precipitated cells gave results which are summarized in Table IX. As may be seen, in both light and dark, the existence of a large quantity of ordinary CO_2 in the gas phase caused altered neither the rate of $C^{14}O_2$-liberation nor the rate of C^{14}-incorporation in bacterial cells.

TABLE IX

Effect of the Presence in Excess of Ordinary CO_2 upon the Distribution of C^{14} in the TCA-precipitated Cells and CO_2 Evolved during the Metabolism of Carboxyl-C^{14}-labeled Lactate

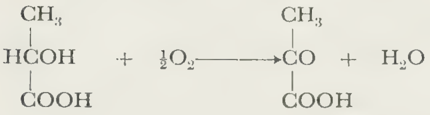
CO_2, 5% ; substrate, carboxyl-C^{14}-labeled lactate, 5 μM, 984 c.p.m. (initial amount) ; bacteria, 10 mg. per vessel ; total volume of suspension, 5 ml.

	Gas phase filled with	Time (min.)	C^{14} incorporated in	
			TCA-precipitated cells	CO_2
		min.	*c.p.m.*	*c.p.m.*
Dark	5% CO_2 in air	30	198	135
		60	319	277
	CO_2 free air	30	185	199
		60	285	296
Light	5% CO_2 in air	30	238	43
		60	598	108
	CO_2 free air	30	249	93
		60	552	118

DISCUSSION

All the data obtained in the present study may be explained by assuming the occurrence of the following three reactions :

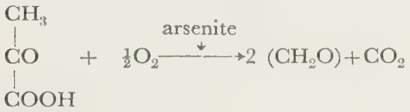
(1) Primary oxidation of lactic acid to pyruvic acid:



$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{HCOH}\\ |\\ \mathrm{COOH}\end{array} + \tfrac{1}{2}\mathrm{O_2} \longrightarrow \begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{H_2O} \qquad (1)$$

which occurs independently of light.

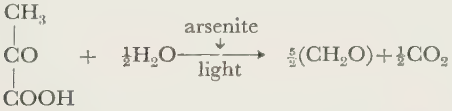
(2) Oxidative breakdown of pyruvate occurring in the dark and not on the light:



$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{COOH}\end{array} + \tfrac{1}{2}\mathrm{O_2} \xrightarrow{\overset{\text{arsenite}}{\downarrow}} 2\ (\mathrm{CH_2O}) + \mathrm{CO_2} \qquad (2)$$

which is very sensitive to arsenite. In this reaction the CO_2 evolved derives solely from the carboxyl group of pyruvate.

(3) Photochemically induced non-oxidative breakdown of pyruvate:

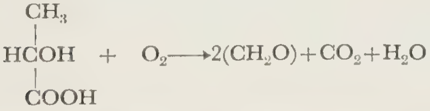


$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{COOH}\end{array} + \tfrac{1}{2}\mathrm{H_2O} \xrightarrow[\text{light}]{\overset{\text{arsenite}}{\downarrow}} \tfrac{5}{2}(\mathrm{CH_2O}) + \tfrac{1}{2}\mathrm{CO_2} \qquad (3)$$

which is less sensitive to arsenite than Reaction (2). Also in this case, CO_2 derives from the carboxyl group of pyruvate. It should be remarked that Reaction (1) may not be quite refractory against the action of arsenite, although its sensitivity is apparently far less than that of Reaction (2).

In the following we shall see to what extent the deductions made from these assumptions are in alignment with the experimental results.

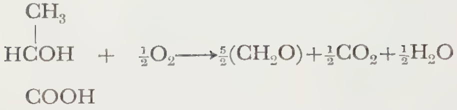
(i) In the dark, lactic acid is metabolized by the sequence of Reactions (1) and (2), with the net result:



$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{HCOH}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{O_2} \longrightarrow 2(\mathrm{CH_2O}) + \mathrm{CO_2} + \mathrm{H_2O} \qquad (4)$$

which corresponds to the findings that in the dark 1 mole of oxygen was absorbed per mole of lactic acid consumed (Table I), and that there occurs no accumulation of pyruvate.

(ii) In the light, lactic acid will be metabolized according to Reaction (1) followed by Reaction (3), therefore:



$$\begin{array}{c} CH_3 \\ | \\ HCOH \\ | \\ COOH \end{array} + \tfrac{1}{2}O_2 \longrightarrow \tfrac{5}{2}(CH_2O)+\tfrac{1}{2}CO_2+\tfrac{1}{2}H_2O \qquad (5)$$

which is in accord with the observation that in the light 1/2 mole oxygen was absorbed per 1 mole of lactic acid consumed (Table I). Accumulation of pyruvate did not occur in this case either.

(iii) When lactic acid is given as substrate, the rate of O_2-uptake in the dark is twice as high as that in the light (Figs. 1, 3, 4 and 6), while the rate of consumption of lactate was not affected by illumination (Table I).

(iv) When pyruvate was given as substrate, no oxygen was taken up in the light (corresponding to Reaction (3), whereas in the dark 1/2 mole oxygen was absorbed per 1 mole pyruvate consumed (corresponding to Reaction (2)), (Tables IV and V).

The fact that the rate of consumption of pyruvate *per se* was not markedly altered by illumination indicates that Reactions (2) and (3), which pyruvate undergoes under different light conditions, proceed with practically the same velocity.

(v) In the presence of a sufficient quantity (10^{-3} M) of arsenite, pyruvate was accumulated as an oxidation product of lactate, and indeed both in the light and in the dark (Table III).

The data given in Fig. 5 shows that Reactions (2) and (3) are inhibited by 10^{-3} M arsenite to an extent of 95 per cent and 25 per cent, respectively. If Reaction (1) is quite refractory to arsenite, the expected inhibition caused by 10^{-3} M arsenite of the O_2-uptake in Reaction (4) and (5) would be 50 and 0 per cent, respectively, instead of 84 and 70 per cent, which was found experimentally. This discrepancy indicates that also Reaction (1) is appreciably sensitive towards the inhibitory action of arsenite.

(vi) According to Reactions (4) and (5), the ratios of the lactic acid consumed (L), oxygen absorbed, CO_2 evolved and the carbon assimilated in the bacterial cells (C) will be

In the dark .... $L : O_2 : CO_2 : C=1 : 1 : 1 : 2$
In the light............... $L : O_2 : CO_2 : C=1 : 0.5 : 0.5 : 2.5$

The values obtained in the experiment using homogeneously C^{14}-labeled lactate are satisfactorily in agreement with these theoretical values, namely:

In the dark.............. $L : O_2 : CO_2 : C=1 : 1 : 1.3 : 2.0$

In the light.............. $L : O_2 : CO_2 : C = 1 : 0.52 : 0.57 : 2.2$

(vii) With the assumption that both in Reactions (2) and (3), the carbon dioxide evolved derives from the carboxyl-group of pyruvate and, therefore, from the same group in lactate when lactate was used as substrate, it is expected from Reactions (4) and (5) that, when carboxyl-C^{14}-labeled lactate was given as substrate, the ratio of radioactivity in carbon dioxide produced and in TCA-precipitated bacterial cells will be:

In the dark .... 1 : 0
In the light .... 1 : 1

This is also in good agreement with the experimental data: 1 :0.17 in the dark, and 1 :0.93 in the light.

These considerations demonstrate that the various observations made in our experiments have received a coherent and rational explanation by the assumption of the Reactions (1), (2) and (3). It should be remarked that, in the equations representing Reactions (2) and (3), the cellular material formed was indicated simply by the formula (CH_2O), which, in fact, involves a large variety of substances as we have shown experimentally. Under the condition of our experiments, the C^{14} assimilated from the homogeneously C^{14}-lacbeled latate was found almost evenly distributed in various fractions of cell constitutens. No doubt, the very primary assimilation product derived from pyruvate must be a substance of difinite chemical species, and presumably it will be different according as the reaction occurs in the dark (Reaction (2)) or in the light (Reaction (3)). In this connection, the fate of the carboxyl-group in pyruvate (or of lactate) is of special interest. We have seen that in the dark it was totally liberated as CO_2 withiout being assimilated, whereas in the light it was half liberated as CO_2 and half incorporated in cellular material. It was confirmed that the assimilation C^{14}-carboxyl-group in the light was not affected by the presence of an excess of unlabeled CO_2 in the atmosphere. Two lines of speculation may be suggested to account for this observation; namely, (1) carboxyl groups out of two molecules of pyruvate will be both liberated as CO_2, and then one of them will be immediately, and for some reason preferably to the CO_2 supplied from outside, photosynthetically assimilated by the same mechanism as the photosynthesis of the ordinary CO_2, or (2) it is assimilated, without being decarboxylated, directly into cell meterial as a part of some organic molecule. The possibility of second assumption seems to be strengthened when we consider the significance of phosphoglyceric acid as a primary intermediate of photosynthesis occuring in green plants. Clarification of this points as well as the identification of the primary assimilation product derived from pyruvate may be achieved by experiments of short duration using C^{14} as a tracer.

The efficiency, with which our bacterium assimilates lactate in the light and dark, deserves special notice. It was shown in our experiments that per mole of lactate (C_3) consumed, 2.2 and 2.03 carbon atoms were assimilated in the light and dark, respectively. Such a high efficiency can hardly be expected in ordinary micro-organisms. So far as we are aware, the activity of assimilating lactate with comparable efficiency has been observed only in *Pseudomanas saccharophylla* studied by Wiame and Doudoroff (*10*).

SUMMARY

1. The effect of light on the metabolism of lactic and pyruvic acids by *Rhodopseudomonas palustris* was investigated.

2. In the dark, lactic acid was metabolized with the consumption of 1 mole oxygen per mole of lactic acid. In the light, only 1/2 mole of oxygen absorbed per mole of lactic acid consumed. The rate of consumption of lactate itself was not affected by light. Both in the light and in dark, no pyruvate accumulated as a product of lactate metabolism. Accumulation of pyruvate was observed in the presence of arsenite, and indeed both in the light and in darkness.

3. When pyruvate was given as substrate, no oxygen was taken up in the light, whereas in the dark 1/2 mole oxygen was absorbed per 1 mole of pyruvate consumed.

4. Using homogeneously C^{14}-labeled lactate as substrate, the ratios of the carbon assimilated in bacterial cells (C) were found to be approximately as follows:

In the dark............... $L : O_2 : CO_2 : C = 1 : 1 : 1 : 2$
In the light............... $L : O_2 : CO_2 : C = 1 : 0.5 : 0.5 : 2.5$

5. When carboxyl-C^{14}-labeled lactate was given as substrate, the ratio of radioactivity in carbon dioxide produced and that incorporated in bacterial cells was found to be approximately as follows:

In the dark............... 1 : 0
In the light............... 1 : 1.

6. All the experimental facts described above were coherently explained by the following assumptions:

(i) Both in the light and dark, lactate is primarily oxidized to pyruvate according to the formula:

$$\text{Lactate}+\tfrac{1}{2}O_2\longrightarrow\text{pyruvate}+H_2O.$$

(ii) In the dark, pyruvate is further oxidized in the following manner:

$$\text{Pyruvate}+\tfrac{1}{2}O_2\longrightarrow 2(CH_2O)+CO_2.$$

The CO_2 evolved in this reaction derives solely from the carboxyl group of pyruvate.

(iii) In the light, Reaction of (ii) is totally suppressed, and instead, the following non-oxidative reaction takes place:

$$\text{Pyruvate}+\tfrac{1}{2}H_2O\longrightarrow\tfrac{5}{2}(CH_2O)+\tfrac{1}{2}CO_2.$$

Also in this case, CO_2 derives from the carboxyl group of pyruvate.

(vi) Arsenite inhibits the above mentioned three reactions in the order:

$$\text{(ii)} > \text{(iii)} \geqq \text{(i)}.$$

7. By using homogenously C^{14}-labeled lactate as substrate, and by subsequently measuring the radioactivity incorporated into various cellular fractions, it was found that, both in the light and in the dark, the assimilated carbon atoms were almost evenly distributed in various fractions of cell materials.

8. The process of lactate metabolism occuring in the light, namely:

$$\text{Lactate}+\tfrac{1}{2}O_2\longrightarrow\tfrac{5}{2}(CH_2O)+\tfrac{1}{2}CO_2+\tfrac{1}{2}H_2O.$$

shows that the carboxyl group of lactate was half liberated as CO_2 and half incorporated in cellular materials. The assimilation of C^{14}-carboxyl of lactate in the light was found not to be affected by the presence of 5 per cent unlabeled CO_2 in the atomosphere. This fact was interpreted as suggesting that a major part of the carboxyl group of lactate was assimilated, without being decarboxylated, directly into cell materials.

This work has carried out under the supervision of Professor Hiroshi Tamiya, to whom the author is indebted for his continual interest. Thanks are due to Dr. A. Takamiya of the Tokyo Institute of Technology, and Messrs. H. Takahashi and M. Nomura, Institute of Applied Microbiology, for their kind help in carrying out this research. This work was supported by the Scientific Research Expenditure from the Ministry of Education, to which thanks are due.

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CONVERSION OF KYNURENAMINE TO KYNURINE BY MOUSE LIVER HOMOGENATE

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(Received for publication, June 4, 1955)

Formerly Makino and Takahashi (*1*) synthesized dimethylkynurenamine hydrochloride and found its lowering action on the blood pressure.

Now the authors intended to synthesize kynurenamine itself in order to make some physiological experiments with it. The synthesis was done by the saccharine method while Butenandt (*2*) *et al.* succeeded in it by the phthalimide method. The starting material which the authors used was *o*-nitro-β-dimethyl aminopropiophenone hydrochloride prepared according to Takahashi (*3*). This was condensed with saccharine to nitrosaccharyl derivative, which was reduced with stannous chloride to the amino compound. This was heated with hydrochloric acid in a sealed tube to eliminate *o*-sulfobenzoyl-group. The resulting kynurenamine showed an activity a little weaker than that of dimethylkynurenamine in the blood pressure test using rabbits as the test animals.

According to Makino and Takahashi (*4*) 5-hydroxykynurenine was converted to 6-hydroxykynurine by mouse liver homogenate. In the early stage of this reaction a substance thought to be 5-hydroxykynurenamine was produced as the intermediate. Makino and Arai (*5*) found that from 3-hydroxykynurenine 8-hydroxykynurine was produced by liver homogenate. The present authors intended to see the behaviour of kynurenamine to mouse liver homogenate and knew that the amine was easily convertible to kynurine, which was proved by paper chromatography and ultraviolet spectrography.

EXPERIMENTAL

Synthesis of Kynurenamine

o-Nitro-β-saccharyl Propiophenone—5 g. of *o*-nitro-β-dimethylamino-propiophenone hydrochloride were dissolved in 50 ml. of water and overlapped with 33 ml. of ether and cooled to 0°. To this mixture, 9 ml. of 2 *N* NaOH were added drop by drop with vigorous shaking, when the mixture turned to a yellow colour. The ether layer was separated and the aqueous layer was washed twice with ether. The ether washings were united to the first ether layer, which was dried with sodium sulphate. To this ether solution 3.5 g. of saccharin were added and the whole was evaporated under the diminished pressure, and 10.3 ml. of absolute alcohol and a small amount of sodium ethylate were added to it and the mixtu:e was refluxed for two hours on the water bath, when the mixture turned black and the added saccharin was dissolved completely. On cooling it turned turbid and deposited a oily matter, which was decanted and, on dissolving in acetone and adding a small amount of methanol, gave needle-like crystals. (m.p. 115°). Yield 2.4 g.

Analysis.	$C_{16}H_{12}O_6H_2S$	Calcd.	C 53.33%	H 3.36%	N 7.78%
		Found	C 53.12%	H 3.59%	N 7.67%

o-Amino-β-sacharyl Propiophenone—1 g. of *o*-nitro-*β*-saccharyl propiophenone was dissolved in 10 ml. of acetic acid by warming and 2.5 g. of stannous chloride dissolved in 3 ml. of concentrated hydrochloric acid were added, resulting a yellow solution, which, on heating on the water bath for 5 or ten minutes and adding 30 ml. of hot water, turned turdid giving gradually a crystalline deposite. After placing in a refrigerator in order to make crystallization completely, yellow needle-like crystals were filtered. (m.p. 174°). Yield 0.7 g.

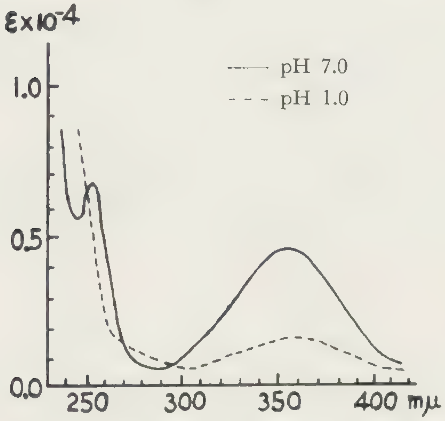
Analysis.	$C_{16}H_{14}O_4N_2S$	Calcd.	N 8.4%
		Found	N 7.8%

Kynurenamine Hydrochloride—0.6 g. of *o*-amino-*β*-saccharyl propiophenone, 3 ml.of glacial acetic acid and 1.8 ml. of concentrated hydrochloric acid were heated in a sealed tube at 150° for 1 hour giving a transparent yellow solution, which deposited on cooling crystalline *o*-sulfobenzoic acid. The mixture was diluted with 10 ml. of water, filtered with glass filter, and the filtrate was concentrated under the diminished pressure giving needle-like crystals, which were filtered and dissolved in 20 ml. of anhydrous alcohol and filtered after the addition of charcoal. The alcoholic solution on standing in a refrigerator gave a crystalline matter, which was filtered. (m.p. 187-188°). Yield 1.0 g.

Paper chromatographic detection showed that this crystal contained some impurities. When this was treated with boiling absolute alcohol, 0.1 gl. of unsoluble matter was obtained, m.p. 188°. Its filtrate on addition of a small amount of ether yielded 100 mg. of needle-like crystal melting at 187°.

On paper chromatography, using a mixture of butanol, glacial acetic acid and water (4 : 1 : 5) as the developer, the sample of m.p. 188° gave Rf of 0.2, the same value as that of the sample obtained according to the phthalimide method, and in accordance with that no melting point depression was seen on admixing both the samples, while the sample of m.p. 187° showed, besides the main spot of Rf 0.2, another faint spot of Rf 0.3, which showed no ninhydrin reaction differing from the compound of Rf 0.2. Its ultraviolet absorption spectrum was indicated in Fig. 1.

Fig. 1



Kynurenamine Picrate—10 mg. of kynurenamine hydrochloride was dissolved in 1 ml. of water to which a solution of 9.66 mg. of picric acid in 0.88 ml. of 1/10 *N* sodium hydroxide was dropped. A yellow needle-like crystal deposited which was filtered and recrystallized from hot water, yielding a red needle-like crystal of m.p. 102° in accordance to the description of Butenandt (*2*).

Cleavage of Kynurenamine with Liver Homogenate

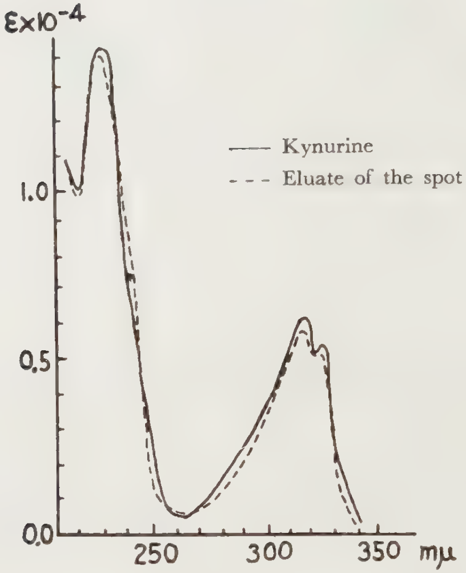
A half mg. of kynurenamine hydrochloride was incubated with 1 ml. of 50 per cent liver homogenate in phosphate buffer (pH 7.2) at 37°, and at the interval of 10,30 minutes, 1 hour, 2 hours and 3 hours, respectively, an aliquot was taken out from the reaction

Table I

Rf Values of Kynurine and Eluate in Various Solvent Systems

Solvent / Substance	Kynurine	Eluate of reaction product
n-Butanol saturated with water.........	0.78	0.78
n-Butanol saturated with 1% NH_3 solution	0.81	0.81
n-Butanol-acetic acid-water	0.81	0.81
n-Butanol saturated with 10% urea solution	0.80	0.80
80% isopropanol	0.82	0.82

Fig. 2



mixture and, without deproteinization, was developed on filter paper with butanol saturated with 10 per cent urea solution. At Rf 0.80 we detected a reaction product with the densitometer for λ_{310}mμ., which was also detectable with 1 per cent ferri chloride solution giving a brown colouration. This substance showed the same Rf values as kynurine in several solvent system as indicated in Table I and the same absorption spectrum as kynurine as indicated in Fig. 2.

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THE SYNTHESIS OF DIMETHYL 5-HYDROXYKYNURENAMINE AND ITS ACTION ON THE BLOOD PRESSURE OF RABBIT

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(Received for publication, June 4, 1955)

Previously we reported that dimethyl kynurenamine had a remarkable blood pressure lowering activity and showed an antagonistic action to the adrenaline hypertension (*1*). After that we knew that kynurenamine had also the same action on the blood pressure, though it was a little weaker than that of dimethyl kynurenamine.

In this paper we will report on the synthesis of dimethyl 5-hydroxykynurenamine and its physiological action.

Dimethyl 5-Methoxybenzoyl Ethylamine—6-Nitro-3-methoxyacetophenon (2.2 g.), paraformaldehyde (10.3 g.) and dimethylamine hydrochloride (1.0 g.) were boiled in butanol (about 1 ml.) for about half an hour and then 50 ml. of dry aceton were added. On cooling in an refrigerator 2.9 g. of white crystal were obtained, which were dried and recrystallized from acetone. (m.p. 208°, m.p. of picrate 141°).

Analysis.	$C_{18}H_{19}O_{11}N_5$	Calcd.	C 45.10,	H 3.88,	N 14.55%
		Found	C 46.04,	H 3.67,	N 14.27%

Dimethyl 5-Hydroxykynurenamine Hydrochloride—Four hundred-sixty mg. of the above compound were dissolved in alcohol with a little acetic acid, and hydrogenated in the presence of palladium charcoal. After removing the solvent in vacuo the residue was boiled with hydrobromic acid (D 1.38, 10 ml.) for about nine hours under carbon dioxide gas and evaporated with hydrochloric acid, giving dimethyl 5-hydroxykynurenamine hydrochloride, m.p. 210–215°.

This compound is easily soluble in water, giving a yellow solution having a green fluorescence. It gives orange colour with *p*-dimethylaminobenzaldehyde in hydrochloric acid, reddish orange colour with Dragendorff's reagent, and purple with the diazo reagent. Its Rf value on paper chromatogram was 0.69–0.70 with 70 per cent *iso*-propanol, 0.60–0.63 with a butanol-acetic acid-water mixture (4 : 1 : 2). Its ultraviolet absorption spectrum has a maximum at 385 mμ in pH 7.0, 382 mμ in pH 5.0. The aquanous solution gave a picrate which was very hygroscopic and tended to become reginous substance.

When various amounts of dimethyl 5-hydroxykynurenamine hydrochloride were injected intravenously to rabbits, the result as indicated in the following table was obtained.

TABLE I

The compound tested	Blood pressure lowering activity	Duration of the activity
μg./kg.	*mm. Hg*	
Dimethyl 5-hydroxykynurenamine		
50	14	18 *sec.*
100	20–22	
200	32	1 *min.* 40 *sec.*
500	40	3 *min.* 50 *sec.*
Dimethyl kynurenamine		
100	15	
200	28	
500	30	

Judging from this result, it seems that the action of dimethyl 5-hydroxykynurenamine is stronger than that of dimethyl kynurenamine.

By interrupting the incubation of 5-hydroxykynurenine with liver homogenate at the early stage and fractionating the cleavage products by paper chromatography we obtained a substance, which was judged to be 5-hydroxykynurenamine from its properties. The diluted solution of this substance showed also the blood pressure lowering action.

This work was aided by a grant from the scientific research fund of the Ministry of Education of Japan. We wish to express our thanks to the Takeda Research Laboratory for performing elementary analysis.

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NUTRITIVE VALUE OR TOXICITY OF HIGHLY UNSATURATED FATTY ACIDS. II

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(Received for publication, June 5, 1955)

In the previous paper (*1*), the authors have established that highly unsaturated fatty acids are not harmful at all when the fatty acids are thoroughly refined so as not to autoxidize themselves. Moreover, it has been found that the widely-believed toxic effects of highly unsaturated acids are primarily caused, not by the unsaturation of fatty acids, but by the autoxidized products which are yielded from highly unsaturated acids.

Since the authors presumed that this conclusion might apply not only to the highly unsaturated acids but to all of the members of natural unsaturated fatty acids, the same course of feeding experiments was carried out, using ethyl esters of liquid acids of linseed oil.

The results of experiment were entirely identical as expected. Based upon these results, it is considered that all the unsaturated acids, which are not easily autoxidized, become harmful once they autoxidize themselves. It was, therefore, tried to examine whether the autoxidized products show toxic effects, by the way of separating such products from other non-toxic ones.

In this case, it was presumed that all the autoxidized products of the unsaturated fatty acids, such as peroxides, conjugated acids, polymerized products, aldehydes, ketones, *etc.* might not be equally toxic but only one or some specific products might be highly toxic and the rest not being toxic or at the least toxic.

In order to verify this presumption, each product was separated and each of them was subjected to the animal test.

When autoxidized slightly, a greater part of unsaturated acids tend to remain for a while with the original structure, showing the most harmful results as stated in our previous report (*1*).

As regard to the method of concentrating the autoxidized products from autoxidized unsaturated acids, several papers have been reported. Privett *et al.* (*2*) separated autoxidized products, using Skellysolve F and ethyl alcohol, the Changs (*3*) did it, using Skellysolve F and diethyl ether. Zilch (*4*), Fugger (*5*), Cannon (*6*), *etc.* separated by countercurrent extraction procedure and Atherton, Hilditch (*7*) did it by chromatographic method using silica gel. Furthermore, in these several years, Catravas (*8*), Coleman (*9*), Swern (*10*) have reported the method of fractionation by urea complexes.

Therefore, these methods were adopted to separate the autoxidized products. Firstly, adopting the Privett's method (*2*), petroleum ether and 85 per cent ethyl alcohol were used for separation. Most of the autoxidized products solved into alcohol and some remained in the part of the petroleum ether.

The both fractions were harmful for the rats.

The experiment means that, autoxidized products were condensed in this method, but the method was not effective to separate the wholly unharmful fraction out of the harmful one as desired by the authors.

The Atherton and Hilditch's chromatographic method (*7*) of using silica gel showed some toxic substances remaining in the fraction without being absorbed by the silica gel, and did not satisfy our object primarily. However, when chromatographic method is applied, using alminium oxide, the fraction not absorved to alminium oxide is totally harmless. But it is difficult to elute fully the absorbed products from the alminium oxide, endangering the materials to be autoxidized further in the course of experiments.

Having tried the separation by urea complexes, 75 per cent of ethyl esters of autoxidized linseed oil produced urea adduct, showing harmless, but the fraction which did not produce urea adduct was quite toxic.

The fraction which produced urea adduct contains unchanged fatty acids of linseed oil, slightly polymerized products, aldehydes, *etc.* and a very little of autoxidized products. On the other hand, the fraction which did not produce urea adduct contains a little aldehyde, showing a high peroxide value and also contains some conjugated fatty acids which were produced by autoxidation.

As a result of the above, aldehyde does not have any conspicuous toxic effect but the actual harmful products in autoxidized fatty acids are some products with branched-chain fatty acids, lactone, polymerized products, conjugated acids and peroxide.

When unsaturated fatty acids are polymerized, nature of the products varies according to the temperature. At the lower temperature peroxide value goes higher, while at the higher temperature it does not go up, as well verified by Atherton and Hilditch as did by the authors.

With highly unsaturated acids polymerized at the higher temperature and decreased iodine value, no toxic effect appeared at the animal test and the rats grew up well. This means the polymerized products of highly unsaturated acids without oxidized product are harmless, and some substances which contain conjugated linkage often turns out to be toxic as has been known well for a long time. The unsaturated fatty acids which autoxidized at the lower temperature, according to our experiment, contains about 6 per cent of conjugated acids, equivalent to 0.03 g. of daily feed for rats. The test of feeding experimentally about 5 times of conjugated acids of tung oil show harmless result.

According to the results gained, conjugated fatty acids are not considered to be the prime cause of producing toxic effect in the autoxidized unsaturated acids.

From the above results, the most harmful fraction contained in autoxidized unsaturated acids must be branched-chain fatty acids, lactone or peroxide.

Judging from these results, the authors assumed that the toxic effects should certainly be attributable mainly to the formation of preoxide-structure.

Supposing the main toxicity is to be attributed to peroxide, autoxidized product, which were freed from peroxide, must be quite harmless or slightly toxic. Then, using the same method as applied to the Lea's peroxide determination method (*11*), potassium iodide and acetic acid were added to the autoxidized liquid esters of linseed oil and freed from peroxide structure, and then checked how harmful the feed was. We found out, as expected, the toxicity was quite weak and the rats did not die (rats had slightly loose bowels).

The same experiment was applied to the autoxidized highly unsaturated fatty acids and the result was also harmless.

In other words, it was confirmed from the above result that the most harmful factor among the autoxidized fatty acids was peroxide structure.

The lethal dose for mice of the peroxide in autoxidized fatty acids was confirmed by the fact that about 278 mg. of total peroxide oxygen per kg. of mice caused the death of the half of the mice tested.

Now, the remaining question is that by what process and mechanism the peroxide in autoxidized acids gives toxicity to the rats.

As the means of inquiring into this question, the authors asked Mr. Saito and Mr. Ushiki of Tokyo University, Medical Department to make sample tips of the internal organs of the rats which were fed with autoxidized acids and about to die. They have found that the tubuli uriniferi of kidney was extended at the border of medulla and cortical layers, and the cell-infiltration was observed at the mucous membrane of small intestine.

Moreover, the mitochondria were isolated from the rats liver with 0.25 *M* saccharose solution, and when one drop of autoxidized highly unsaturated acids added to half of this solution, the number of mitochondria decreased quickly. On the other hand, when the peroxide-free acids were added to another half, the mitochondria did not decrease. And we observed that the peroxide was found from fats of liver and muscle of rats when autoxidized unsaturated acids were fed.

Having concluded from the above results obtained, peroxide in the autoxidized fatty acids might directly affect the digestive organs of rats, or indirectly might be absorbed, through the rats digestive organs, into the rats organs and give toxic effects to them by the peroxide itself or its secondary disintergrated product.

EXPERIMENTAL

Feeding Procedures—Weanling male and female rats obtained from our stock colony were depleted by feeding them with 9.5 g. of the fat-deficient diet per day per rat. When their body weight remained almost constant, they were separated into different groups.

TABLE I

Composition of Diets

Ingredients	Amount (%)	Ingredients	Amount (mg./100 g. diets)
Polished rice powder	83	Vitamin A	80 (U.S.P.U.)
Casein (ether extracted)	10	Tocopherol	3
McCollum salts mixture	3	Thiamine	0.125
Sample lipides	2–5	Rivoflavin	0.1
		Pyridoxine	0.1
		Pantothenic acid	1
		Niacin	1.5
		Choline	100
		Inositol	200
		p-Aminobenzoic acid	0.5
		Vitamin B_{12}	0.0015

0.2–0.5 g. of sample lipides were daily administered per rat over a 30-day period to observe growth and other physiological conditions of these animals.

Experiment I

Preparation of Ethyl Ester of Liquid Acids of Linseed Oil—The mixed fatty acids of linseed oil were dissolved in aceton, fractionally crystallized in cold storage and separated the solid acids portion. The aceton of filtrate was distilled under vacuum. The obtained liquid acids were changed into ethyl ester in the presence of HCl-catalyst, and subjected to the vacuum distillation. Then the sample was purified by chromatography of alminium oxide.

The sample of autoxidized acids was prepared by leaving a 4 mm. layer of genuine liquid acids of linseed oil in an open basin at 30°. 50 hours, and 100 g. of this autoxidizes liquid ester was added to a hot solution of 700 g. urea in 1000 ml. of methanol. A precipitate was formed immediately, but the mixture was heated to effect complete solution, and then it was allowed to cool to the room temperature. The well-defined needles of urea complexes were filtered with suction (Fr. 1), and the filtrate was then dissolved to 85 per cent methanol solution and separated the petroleum ether soluble fraction (Fr. 2). Moerover, the petroleum insoluble fraction was extracted with ether (Fr. 3).

TABLE II

Diagram showing Scope of Fractionation of Autoxidized Liquid Acids of Linseed Oil by Urea Adduct Formation

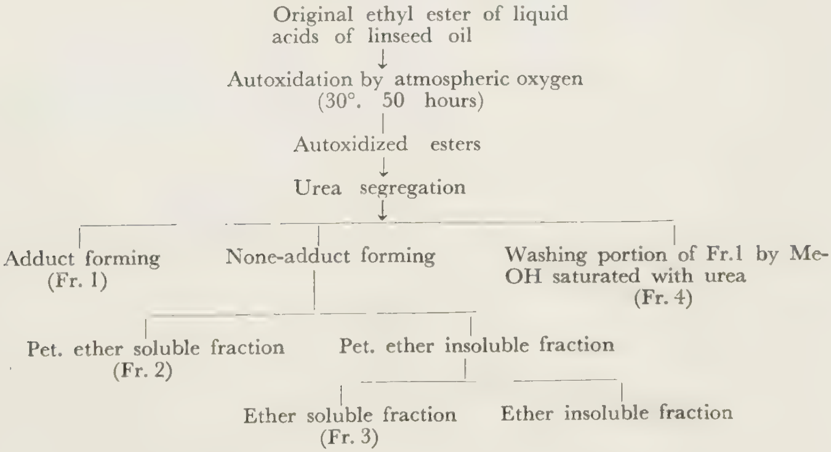
Original ethyl ester of liquid acids of linseed oil
Autoxidation by atmospheric oxygen (30°. 50 hours)
Autoxidized esters
Urea segregation
Adduct forming (Fr. 1)
None-adduct forming
Washing portion of Fr.1 by MeOH saturated with urea (Fr. 4)
Pet. ether soluble fraction (Fr. 2)
Pet. ether insoluble fraction
Ether soluble fraction (Fr. 3)
Ether insoluble fraction

TABLE III

Analysis of the Samples Used in the Experiments

Sample ester	Yield (%)	Acid value	Sap. value	Iodine value	Peroxide value (S.C./kg.)	Kreis test	Conjugated acid (%)		
							Diene	Triene	Tetra-ene
Original ethyl ester of liquid acids of linseed oil		0	176.01	168.28	0	—	4.97	0	—
Autoxidized esters of above acids		6.4	184.85	137.45	409	⊕	6.07	0.08	0
Fr. 1	74.9	3.5	177.30	143.44	33	⊕	1.01	0	—
Fr. 2	6.1	14.8	213.20	116.43	1216	⊕	30.15	0	0
Fr. 3	11.9	3.8	242.97	77.53	1585	⊖	17.63	0.69	—
Fr. 4	3.7		223.43	106.91	1203	—			—
Total	96.6								

TABLE IV

Feeding Records of Autoxidized Esters of Linseed Oil

(Period: From Mar. 16 to April 5, 1954)

Type of fat fed	Brood series	Sex	Body weight (g.)		State of mortality
			Initial	Last day	
Autoxidized esters of linseed oil, 0.5 g.	C	♀	49	40	Died on 4th day
	C	♀	41	30	" 3rd "
	A	♀	39	44	" 3rd "
	A	♂	38	37	" 7th "
Fr. 1, 0.4 g.	C	♀	46	56	Survived over 20 days
	C	♀	48	64	" " "
	A	♂	51	79	" " "
Fr. 2, 0.2 g.	C	♀	46	46	Died on 2nd day
	C	♀	39	39	" " "
	A	♂	53	53	" " "
Fr. 3, 0.2 g	C	♀	48	48	Died on 2nd day
	C	♀	46	46	" " "
	A	♀	48	43	" 6th "
	A	♂	52	52	" 2nd "

From these results, it was made clear that the original autoxidized ester and Fr. 2 or Fr. 3 which did not form urea adduct showed the toxic effects upon the growth of rats, and they all died in 7 days. However, Fr. 1 (consisting mainly of un-autoxidized acids) did not give any retarding action to the rats.

Experiment II

Polymerized Ester of Highly Unsaturated Fatty Acids—Genuine ethyl ester of highly unsaturated acids are polymerized in the presence of Japanese acid clay at 120°, 6 hours in carbone dioxide. The properties of the samples were as follows:

TABLE V

Analysis of the Samples Used in the Experiments

	N_D^{40}	Iodine value	Sap. value	Peroxide value (m.m./kg.)
Ethyl ester of oleic acid (control)	1.4440	82.70	184.25	
Original ester of highly unsaturated fatty acids	1.4846	348.79	158.14	11
Polymerized ester of above acids	1.4854	310.62	156.23	62

TABLE VI

Feeding Record of Polymerized Ester of Highly Unsaturated Acids

(Period: April 16 to May 1, 1954)

Type of fat fed	Sex	Body weight gained in 15 days	Mean weight gained
None (Fat-free basal)	♀	8 g.	8 g.
	♀	8	
Ethyl ester of oleic acid (Control), 0.5 g.	♀	16	13
	♀	10	
Original ester of highly unsaturated acid, 0.5 g.	♀	19	17.5
	♂	17	
	♀	16	
	♀	18	
Polymerized ester of above acids, 0.5 g.	♀	20	18.7
	♀	19	
	♀	19	

Polymerized highly unsaturated acids show the nutritive effects to the rats and no retarding effects are noticed.

Experiment III

Nutritive Value of Peroxide-free Products of Autoxidized Unsaturated Fatty Acids—Autoxidized esters of unsaturated fatty acids are weighed into test tube, and the same weight of powdered potassium iodide is added. After adding glacial acetic acid and chloroform (2:1 by volume) 20 times as much as the quantity of the sample ester, carbon dioxide passed into the air space above the liquid to displace most of air, and the tube is heated. The peroxide-free products thus obtained are washed with sodium thiosulfate solution and water, then chloroform is distillated away *in vacuo.*

TABLE VII

Properties of Peroxide-free Products from Autoxidized Liquid Acids of Linseed Oil or Autoxidized highly Unsaturated Fatty Acids

	Iodine value	Sap. value	Peroxide value (S.C./kg.)
Original autoxidized liquid ester of linseed oil	137.45	184.85	415
Peroxide-free products of above acids	136.28		45
Original autoxidized ester of highly unsaturated acids	291.00	179.87	485
Peroxide-free products of above acids	301.04	170.64	33

TABLE VIII

Feeding Records of Peroxide-free Products of Autoxidized Linseed Ester

(Period: Mar. 26 to April 15, 1954)

Type of fat fed	Sex	Body weight		State of mortality
		Initial	Last day	
Autoxidized liquid ester of linseed oil, 0.5 g.	♀	50 g.	50 g.	Died on 2nd day
	♂	61	61	„ 3rd „
	♀	89	94	„ 11th „
Peroxidized-free products of above acids, 0.5 g.	♂	53	55	Survived over 3 weeks
	♀	50	50	„ „
	♀	91	100	„ „

TABLE IX

Feeding Records of Peroxide-free Products of Autoxidized Highly Unsaturated Fatty Acids

(Period: May 24 to June 11, 1954)

Type of fat fed	Sex	Body weight		State of mortality
		Initial	Last days	
Non (Fat-free basal)	♂	$76^{g.}$	$92^{g.}$	Survived over 18 days
	♀	67	77	,, ,,
Autoxidized ester of highly unsaturated acids 0.5 g.	♂	72	72	Died on 7th day
	♀	73	70	,, 4th ,,
	♀	71	61	,, 3rd ,,
Peroxide-free products of above acids 0.5 g.	♂	77	96	Survived over 18 days
	♀	71	84	,, ,,
	♂	75	84	,, ,,

As it is obvious from Tables VIII and IX, peroxide-free products from autoxidized unsaturated fatty acids become non-toxic, and showed nutritive effects upon the growth of rats.

Moreover, linoleic acid was isolated from mixed fatty acids of soybean oil by urea complexes. (The yield of linoleic acid was 87.5 per cent.) Then we carried out the same experiment on the autoxidized liquid acids of linseed oil or highly unsaturated acids, and the same result was obtained.

From these results it has come to the conclusion that the most toxic structure in autoxidized fatty acids is peroxide.

Experiment IV

Lethal Dose of Peroxide Oxygen in Autoxidized Unsaturated fatty Acids to Mice—In order to survey the degree of the toxicity of peroxide produced in unsaturated fatty acids, 0.15–0.5 g. of autoxidized highly unsaturated acids (peroxide value 448 S.C./kg.) was given to mice and estimated the lethal dose of peroxide oxygen against mice.

TABLE X

Lethal Dose of Peroxide Oxygen in Autoxidized Highly Unsaturated Acids Against Mice

Total peroxide oxygen in samples fed to mice *mg.*	1.79	3.58	5.017	7.16
Mortality	0/12	2/12	6/12	10/12

From these results, the lethal dose was not estimated accurately, however, if calculated with 5.017 mg. peroxide oxygen, the LD_{50} is 278 mg. of total peroxide oxygen per kg. of mice.

Experiment V

Peroxide Content of Fats Separated from Liver and Muscle of Rats which Were Fed with Autoxidized Unsaturated Fatty Acids—In order to investigate the origin of toxicity of autoxidized unsaturated acids, the authors determined the peroxide contents of fats of liver and muscle of rats when the autoxidized acids were fed.

The rats which received 0.5 g. of autoxidized fatty acids per day per rat were killed when the retarding action was noticed. The liver and muscle of rats were separated from rats, treated with Na_2SO_4 and the fats were extracted with diethyl ether.

Table XI

Peroxide Value of Fats Separated from Liver and Muscle of Rats which Were Fed with Autoxidized Unsaturated Fatty Acids

Type of sample oil	Peroxide value of sample oil (S.C./kg.)	Peroxide value (S.C./kg.)	
		Liver oil	Muscle oil
None (Fat-free basal)			3
Ethyl ester of oleic acid (control)	0		2
Original ester of highly unsaturated acids	40		14
Autoxidized products of above acids	1405		37
Slightly autoxidized ester of liquid acids of linseed oil	409	20	
Autoxidized ester of liquid acids of linseed oil	3170	89	

No peroxide was found from tissues of the controlled rats which received unautoxidized oleic acid, however, rats fed with autoxidized unsaturated fatty acids contained a large quantity of peroxide in liver and muscle.

DISCUSSION

The survey of the above results leads us to assume that both autoxidized highly unsaturated fatty acids and autoxidized unsaturated fatty acids are all harmful for the rats, and we confirmed that the toxicity is actually caused by the peroxide product.

According to the Duboulozes (*12*), the half of the peroxide was found in the digestive tract of the rats 1.5 hours after the feeding was given to the rats through their mouths.

The authors as well confirmed that considerable volume of peroxide was in the muscle of the rats after autoxidized acids were fed to them.

Based upon these results, it might possibly be considered that the toxicity caused on the rats could be sorted into the direct action which the peroxide gives to the rat's digestive tract or their other internal systems and the indirect one which the absorbed peroxide itself or its secondary disintegrates give toxic effects to tissues.

In other words, just as reported in the previous paper that the rats which were contacted with the autoxidized acids exhibited a depilation around their mouths and legs and also some inflammation, the autoxidized acids are considered to affect the rat's internal systems, attesting it by the fact that the small intestines were injured. Autoxidized acids accumulated in the rat's liver and muscle, which as previously verified, tend to decrease mitochondria in the livers, are assumed to work upon various enzymes, resulting the decrease of enzyme function. However, further research should be made in this respect.

SUMMARY

In the previous paper it has been established that the nutritive value of highly unsaturated fatty acids is not much lower than that of oleic acid, and that generally-believed toxicity of highly unsaturated acids is not produced by the acids themselves but actually by the formation of autoxidized matters.

The observation led us to an assumption that natural unsaturated fatty acids, even if different in the autoxidation degree, would always become less nutritious by some degree of autoxidation.

The assumption was confirmed by the toxic effect on rats of the autoxidized product which was prepared from the ethyl ester of liquid acids of linseed oil, as the rats all died in a few days after the feeding.

On the basis of these findings, our efforts have been extended to throw light on the nature of the above-mentioned toxic product. The method used for and the result obtained from the present test are as follows:

1. The autoxidized products were prepared by leaving the original

ethyl ester of liquid acids of linseed oil, about 4 mm. deep in basin, open to atmospheric oxygen under 30°. for 50 hours.

2. As shown in Table II, the autoxidized products were fractionated by urea complexes, and the nutritive or retarding effects of each fraction were examined.

3. Fr. 1 separated from the autoxidized products did not give any retarding actions to the rats ; while Fr. 2 and Fr. 3 forming urea complexes showed the toxic effects on the animals. From these results it is apparent that aldehydes have no harmful effect upon the rats.

4. On the other hand, polymerized highly unsaturated fatty acids containing a small proportion of peroxide showed the nutritive effects on the rats, but no retarding effect has been discovered.

5. Judging from these results, the toxic effects which were yielded from the autoxidized acids should be attributable mainly to the production of peroxide-structure. In order to prove this assumption, the peroxide of autoxidized products was eliminated by Lea's peroxide determination method. In consequence, peroxide-free products became non-toxic. And the same result was obtained of peroxide-free products of autoxidized highly unsaturated acids.

On the basis of these results, it has come to the conclusion that the most toxic product of autoxidized unsaturated fatty acids is peroxide which has been produced at the biginning of autoxidation.

6. The lethal dose of peroxide oxygen against mice was about 278 mg. of total peroxide oxygen per kg. (LD_{50}).

7. The peroxide was found in liver and muscle fats of rats when the autoxidized unsaturated acids were fed, and if the fat fed to rats contained a large quantity of peroxide, the peroxide value would become higher than that of un-autoxidized acid to rats.

8. The number of mitochondria separated from rats liver was decreased when autoxidized fatty acids were added to the mitochondria solution *in vitro*.

From these findings, it was concluded that the toxicity of peroxide contained in autoxidized unsaturated fatty acids was apparently produced as the result of injuring the tissue of rats.

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CHANGE OF NUCLEIC ACID CONTENT IN CHLORELLA CELLS DURING THE COURSE OF THEIR LIFE-CYCLE

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It has previously been reported that the cells of *Chlorella* assume two distinct forms, " dark cells " and " light cells," in the course of their growth (Tamiya *et al.*,) (*1*). The dark cells are smaller in size, richer in chlorophyll, and stronger in photosynthetic activity than the light cells, and when illuminated, they increase in mass and turn into the light cells. The latter, when ripened, bears a number of autospores which then become individual dark cells. The transformation of light cells into dark cells occurs only under aerobic condition, irrespective of whether the cells are in the light or in the dark. Thus, the over-all process of algal growth is accomplished by the repetition of change between these two kinds of cells as formulated below:

$$D \xrightarrow{\text{light}} L \xrightarrow[\text{of light}]{\text{independently}} nD$$

in which D and L represent, respectively, a dark cell and a light cell, and n the number of dark cells produced from one light cell.

An advantage of using *Chlorella* as a material for biochemical studies on the growth process of algal cells lies in the fact that we can, by adequate procedures, realize a " synchronous growth " of the whole population in the culture, *i.e.*, we can obtain cultures, in which practically all existing cells grow and divide closely keeping pace with each other. By the analysis of the bulk of synchronously growing cells, we can follow the biochemical phenomena occurring in the life-cycle of individual cells, which is otherwise difficult to investigate owing to the smallness of a single cell or the unevenness of the pace of growth shown by individual cells in the bulk of algal population.

The object of this investigation was to examine, using the technique of synchronous culture, the fates of pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) during the course of life-cycle shown by *Chlorella* cells.

METHODS

Culture——*Chlorella ellipsoidea* was cultivated in a flat flask, using the culture medium described in the previous paper (*1*). The inoculum for the main culture was obtained by illuminating the culture with strong light (*ca.* 10,000 lux) at 25° for a few days and then with weak light (*ca.* 800 lux) for about a weak. The algal population thus grown was composed of 80–100 per cent dark cells, whose percentage could be increased by fractionating centrifugation.

The dark cells obtained as above were transferred into 4.5 liters of fresh culture solution contained in a flat culture chamber (5 liters in capacity) having plane-parallel walls (2 cm. in distance) made of lucid acrylate resin (Fig. 1). The chamber was immersed in a water thermostat having a glass wall on one side, and from outside of the wall the culture was illuminated by twelve reflector flood lamps of 300 watt each. The light intensity at the surface of the culture chamber was about 15 kilolux. The thermostat was constantly supplied with thermostated water which was drained along the surface of the glass wall to prevent the heating of the thermostat. The temperature of the culture was thus kept constant at 16°. By means of three glass tubes, air containing 5 per cent CO_2 was constantly bubbled through the cell suspension, by which the cells were kept evenly suspended in the medium. In order to avoid the mutual shading of cells in the suspension, the population density at the beginning of culture was made below 0.3 ml. in packed cell volume per liter.

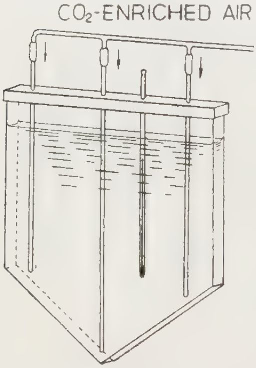

FIG. 1. Culture chamber for growing algal cells synchronously.

With the progress of culture, the algal cells increased in total mass as well as in the size of individual cells—as it is reflected in the increase of packed cell volume per liter of culture, without, however, increasing the cell number. On continued culture in the light, when almost all existing cells turned into fully ripened light cells, they suddenly began to divide giving rise to new dark cells, a phenomenon which was manifest in the increase of cell number and decrease in cell size. At various stages of such a culture, a certain portion of the cell suspension was taken out, and the cells were analyzed for their contents in nucleic acids, total nitrogen and phosphorous, and protein nitrogen.

In order to test the capacity of cells for division in the dark, and to investigate the possible change of cell constitutents during the dark incubation, aliquots of cell suspension taken out at each sampling were transferred into a large test tube and, under constant aeration with air, they were placed in complete darkness at 20°. After standing for 70 hours under such condition*, the cell samples were tested for changes in cell number and in contents of nucleic acids, *etc.*

Determination of Nucleic Acid—PNA and DNA were extracted and assayed spectrophotometrically as described by Ogur and Rosen (*2*) with minor modifications. Centrifuged fresh algal cells were first treated with 90 per cent ethanol and extracted successively with (i) 70 per cent ethanol, (ii) 70 per cent ethanol containing 0.1 per cent percholoric acid, (iii) a mixture of ethanol and ether (3:1) (repeated twice under gentle boiling), and (iv) 0.2 *N* perchloric acid (three times). From the residue, the PNA-fraction was obtained by extraction with cold 1 *N* perchloric acid for 40 hours, and then the DNA-fraction was separated by repeating twice the extraction with 0.5 per-cent perchloric acid at 70° for 20 minutes each. The quantity of nucleic acid in each fraction was determined by measuring the difference between the extinctions at 260 mμ and 310 mμ. Preliminary experiment made with purified preparations of PNA (from yeast) and DNA (from fish sperm), of which also the contents of phosphorus were determined by Allen's method (*3*), showed that the extinction per mole of phosphorus in the nucleic acids used as the standard was 1.14×10^4 in PNA and 9.70×10^3 in DNA**. These factors were used in calculating, from spectrophotometric readings, the contents of PNA-phosphorus and DNA-phosphorus in *Chlorella* cells.

Table I

Spectrophotometric Determination of PNA- and DNA-phosphorus Using Intact and Homogenized Algal Cells of Different Quantity

		Homogenized cells (*a*)	Intact cells (*b*)	*a*/*b*
Dry weight of sample used	*mg.*	571	28.6	20
PNA-P	γ	621	30.8	20
DNA-P	γ	263	14.3	18
$\frac{\text{PNA-P}}{\text{DNA-P}}$		2.4	2.2	

The extractability of each nucleic acid from fresh algal cells and the reliability of spectrophotometric determination were tested by using two samples taken from the same culture– namely, (a) 571 mg. (in dry weight) of cells which were frozen with solid carbon dioxide and ethyl ether, and then finely homogenized before they were subjected to extracting procedure, and (b) 28.6 mg. (in dry weight) of fresh cells which were directly subjected to extracting procedure. As may be seen from Table I, the results of determination in both cases coincided satisfactorily with each other*. Based on these findings, the determinations in the main experiments were carried out by using about 15–30 mg. (in dry weight) of fresh cells.

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* In a preliminary experiment it was confirmed that the light cells, when they were capable of division in the dark, completed their cell division within about 20 hours at 15°–20°. The incubation for 70 hours was, therefore, of sufficient length for testing the capacity of cell division in the dark.

** This value was defined by $30.98 \times \Delta E/c.d.$, wehre ΔE is the difference between the extinctions (log I°/I) at 260 mμ and at 310 mμ, c the concentration of phosphorus in g per liter, and d the thickness of solution in cm.

Determination of Total Nitrogen and Protein-nitrogen—The content of total nitrogen was determined by the micro-Kjeldahl method using about 80 ml. (packed volume) of cells which were washed three times with distilled water before analysis. The bulk protein-nitrogen was determined by the same method using the samples which had been extracted five times with 70 per cent ethanol.

Determination of Total Phosphorus—Total phosphorus was determined according to Allen's method (*3*) using 10–20 ml. (packed volume) of algal cells.

Determination of Cell Number, Packed Cell Volume and Average Cell Volume—The methods used for determining the cell number and packed cell volume per liter of culture were the same as reported earlier (*4*). The ratio (packed cell volume) / (cell number) was regarded as representing the average volume of individual cells. The values thus calculated were found to be more or less larger than those reported earlier, which have been determined by actually measuring the size of 200–300 cells under the microscope.

Determination of " Multiplication Number " (n)—On incubation of light cells in the dark for a sufficient length of time (70 hours), they divide into a number (n) of dark cells. To determine this number, the statistical distributions of cell size before and after the dark incubation were measured. If, during the dark incubation, the number of light cells decreased by ΔL, and the number of dark cells increased by ΔD, the value of n may be represented by ΔD/ΔL. In Fig. 2 and Table II, an example is presented showing the changes in cell number and the distribution of cell size which occurred on incubation in the dark of a culture consisting mostly of well-ripened light cells.

RESULTS

(1) *The Time Course of Synchronous Growth as It Reflected in Changes of Packed Cell Volume, Cell Number and Average Cell Volume*—A synchronous culture started from a dark-cell-rich culture showed the growth curves as shown in Fig. 3. In this figure, open circles (packed cell volume), triangles (cell number), and squares (average cell volume) show the values obtained during the progress of culture kept under illumination,

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* For Sample *a*, phosphorus and sugar contents in both the fractions of DNA and PNA were determined, and the quantities of nucleic acids calculated on the basis of phosphorus and sugar contents were compared with the values obtained by spectrophotometric method. Satisfactory coincidence between these values was found for DNA, while for PNA a considerably higher values were obtained from the measurement of phosphorus or sugar content. It was found that the PNA-fraction contained labile Δ7-phosphate, which showed the metachromatic color reaction with toluidine blue characteristic for inorganic metaphosphate (Wiame (*5*)).

TABLE II

Distribution of Cell Diameter Before and After Incubation of a Light-cell-rich Culture in the Dark

The light and dark cells were distinguished according as the cell diameter was longer or shorter than 4.5 microns. In this example: $\varDelta D = 34.92 - 0.63$, and $\varDelta L = 8.22 - 1.46$, therefore, $n = \varDelta D / \varDelta L = 5.07$.

	Cell diameter in microns	Number of cells per liter ($\times 10^9$)	
		Before incubation in the dark	After incubation in the dark
Dark cells	2.06	0	1.89
	2.16	0	15.57
	3.16	0.21	14.41
	3.71	0.35	2.62
	4.26	0.07	0.43
		Total: 0.63	Total: 34.92
Light cells	4.81	0.43	0.29
	5.36	1.49	0.73
	5.91	2.59	0.44
	6.46	2.16	0
	7.01	0.88	0
	7.56	0.67	0
		Total: 8.22	Total: 1.46

while solid triangles and squares represent the values which were obtained when the cultures at respective stages were incubated in the dark for 70 hours. As may be seen from the figure, the packed cell volume (in ml. per liter) increased throughout the course of illuminated culture, while the cell number remained unchanged up to the 45th hour, after which it suddenly increased to attain a new steady level at about the 62nd hour. Correspondingly, the average cell volume gradually increased up to the 39th hour, and after remaining at a steady level for a short time, it suddenly began to decrease at the 45th hour, and eventually attained the initial low level. One cycle of the synchronous growth was thus completed in about 60 hours, with a result that the total cell number increased about five fold.

It should be remarked that during the period from the 39th to 45th hour the increase of packed cell volume became temporarily sluggish and the average cell volume changed also only slightly. The same phenomenon has been observed in many similar experiments,

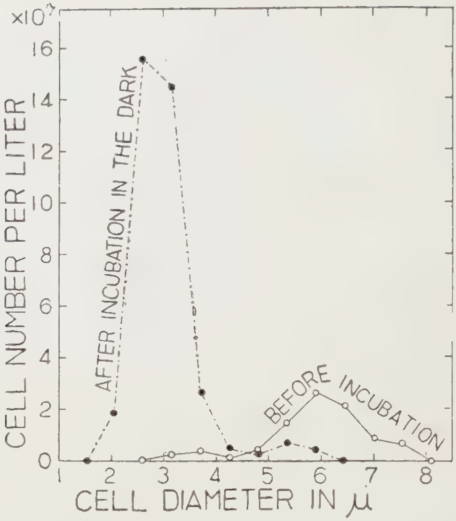

FIG. 2. Distribution curves of cell diameter observed before and and after the dark incubation of light cells.

Continuous curve indicates the number of cells of different sizes which existed before the dark incubation, while the broken line represents the number after the dark incubation.

and it has been shown by Nihei *et al.* (*6*) that at this stage the CO_2-fixation and assimilation were considerably decreased. By microscopic observations it was revealed that the light cells at this stage were characterized by their distinct formation of autospores or their precursors within the cells. This stage may, therefore, be called " sporulating phase " and distinguished from the preceding " growing phase " and the subsequent " dividing phase," the former being the stage of transformation of dark cells into light cells, and the latter the stage of division of the well-ripened light cells into a number of dark cells.

The difference in the nature of cells at these three stages became more striking when the cells were transferred into darkness and kept aerated for 70 hours at 20°. As may be seen from Fig. 3 (solid triangles and squares), the cells at the " sporulating phase " divided completely or almost completely in the darkness, while the cells in the " growing phase " could divide only partially at their later stages. The cells at the " dividing phase " also showed limited capacity for cell division, because they were composed mostly of dark cells produced from the

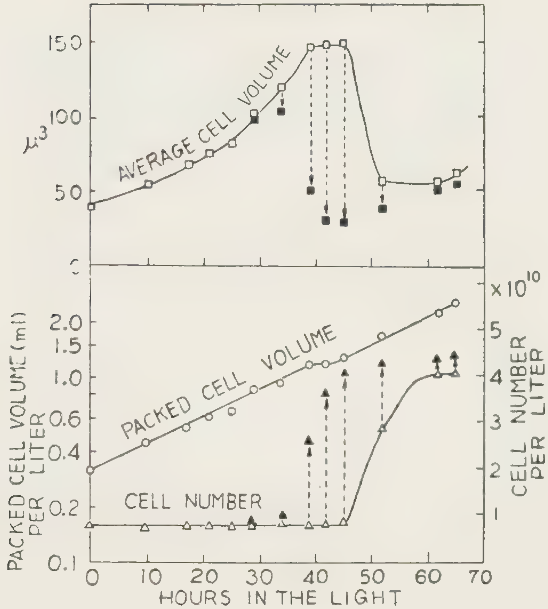

FIG. 3. Change of packed cell volume, cell number and average cell volume during the transformation of dark cells into light cells and vice versa.

Open circles, triangles and squares show the values obtained during the progress of culture kept under illumination, while solid triangles and squares represent the values which were obtained when the cultures at respective stages were incubated in the dark for 70 hours.

light cells.

It is interesting to note that according to the stage of cell development not only the percentage of cells which were capable of division in the dark, but also the "multiplication number" (*n*) changed as it is apparent from the figures given in Table III. The multiplication number shown by the well-ripened light cells on their dark incubation were approximately equal to the corresponding value (5.0) shown by the same cells which had been allowed to divide under continued illumination. This fact indicates that the light had no effect upon the cell division of the well-ripened light cells.

(2) *Pentose-Nucleic Acid and Desoxypentose-nucleic Acid*—During the whole course of the synchronous growth, there occurred characteristic

TABLE III

The Capacity of Cells for Dividing in the Dark as It Changed According to the Stage of Cell Development

Phase of cell development	Length of culture in the light	Percentage of cells capable of dividing in the dark	Multiplication number
	hrs.	%	
Growing phase	0-29	0	1.0
	34	29	1.3
Sporulating phase	39	60	4.1
	42	88	5.1
	45	94	5.3

changes in the contents of PNA and DNA, which are illustrated graphically in Figs. 4, 5 and 6. In respect to the content per liter of algal culture, PNA increased steadily, approximately keeping pace with the increase of packed cell volume, whereas DNA remained almost constant at the beginning of the growing phase and later increased only gradually, until it suddenly increased at the sporulating phase. (Fig. 4) Worthy of special notice is the fact that on incubation of cells in the dark, PNA decreased, gradually to greater extent at later stages of culture, and DNA increased, especially markedly at the sporulating phase. The situation is also illustrated by the curves in Fig. 5 which show the change of percentages (on a dry weight basis) of PNA-P and their ratio during the course of cell transformation. As may be seen, the ratio PNA-P/DNA-P increased during the growing phase, and decreased during the sporulating phase.

An interesting fact emerged when we followed the quantity of DNA-P contained in single cells. In Fig. 6 open and black circles indicate, respectively, the DNA-P-content per cell before and after the dark incubation, which were calculated on the basis of actually found cell number, whereas the solid triangles represent the values obtained by dividing the quantity of DNA-P found *after* the dark incubation by the number of cells observed *before* the dark incubation. The latter values represent, therefore, the DNA-P-content of cells just before their division in the dark. The sharp maximum at the 45th hour shown by the curve connecting the open circles corresponds to the climax of ripening of light cells (in the light). By comparison of the data shown by the open circles with those by solid triangles it may be seen that during the

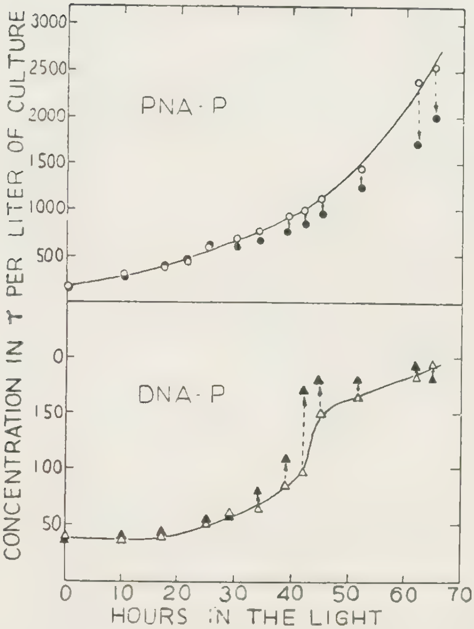

FIG. 4. Change of the quantity of PNA-P and DNA-P (per liter of cell suspension) during the course of cell transformation.

Open circles and triangles represent the values obtained during the progress of culture kept under illumination, while solid circles and triangles show the values which were obtained when the cultures at respective stages were incubated in the dark for 70 hours.

dark incubation the DNA-content per cell increased in light cells, but not in dark cells. The highest level of the solid triangles (*i.e.*, the DNA-content of light cells which became fully ripened in the dark) is about 5 times that of the black circles (*i.e.*, the DNA-content of dark cells produced from light cells), indicating the proportionality existing between the DNA-content and the number of nucleus. The fact that the well-ripened light cells were multi-nucleated, while dark cell were uni-nucleated was confirmed by staining the cells with Feulgen's reagents. Under the microscope it was observed that most of the light cells contained 4 nuclei, and relatively few of them had 2 or 8 nuclei.

(3) *Total Nitrogen, Protein Nitrogen and Total Phosphorus*—With the same culture as described above, the contents in cells of total nitrogen,

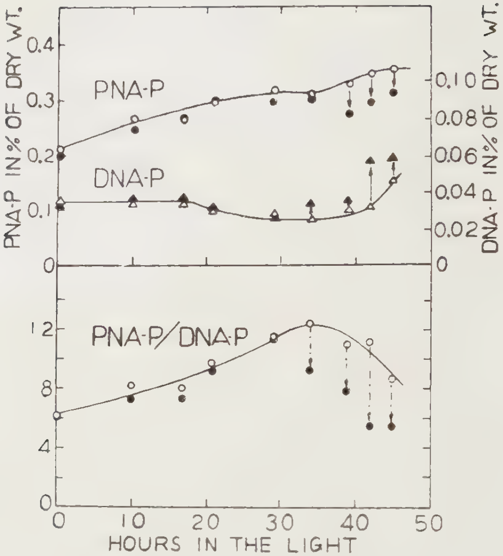

FIG. 5. Percentage (on a dry weight basis) of PNA-P and DNA-P and their ratio as they changed during the course of cell transformation. Open and solid circles or triangle represent, respectively, the values found before and after incubation of cells in the dark.

protein nitrogen and total phosphorus were determined at various stages of cell transformation. The results obtained are presented in Fig. 7. As may be seen, the contents (in percent of dry weight of cells) in total nitrogen and protein nitrogen remained almost unchanged during the growing phase, and increased slightly at the sporulating phase. An abrupt increase at the sporulating phase was also observed in phosphorus content, a fact which may have some connection with the sudden increase of DNA content at that stage. It should be remarked that the contents in total nitrogen, protein nitrogen and phosphosus, unlike those of DNA and PNA, were not modified by the incubation of light cells in the dark.

DISCUSSION

Considering that our experimental material Chlorella represents a sort of prototype of green plant, it may not be amiss to say that the

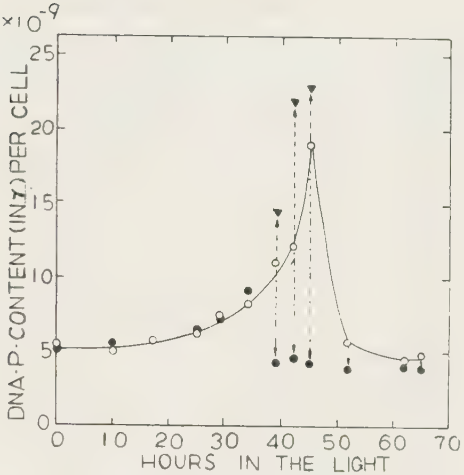

FIG. 6. DNA-P content per cell as it changed with the progress of culture. Open and solid circles represent, respectively, the values found before and after incubation of cells in the dark. The solid triangles represent the values obtained by dividing the quantity of DNA-P found after the dark incubation by the number of cells observed before the dark incubation.

observations made with such an organism give useful suggestions concerning the growth physiology of green plants in general. One of the important observations made in the present study was that the two forms of nucleic acids, PNA and DNA, are synthesized in essentially different ways during the course of algal growth. While PNA is formed during the growing phase, keeping closely pace with the increase of other cellular materials, DNA is produced most actively in the sporulating phase, lagging considerably behind PNA in its formation in the growing phase. Relative constancy of PNA- and protein-contents during the course of cell transformation seems to concur with the prevailing idea that PNA plays an essential role in the process of protein synthesis.

Noteworthy is the fact that on incubating the algal cells in the dark, PNA decreased, while DNA increased, especially markedly in the sporulating phase. The reciprocity in the change of DNA- and PNA-contents during the period of dark incubation suggests strongly that the latter might have been formed by conversion of the former. The data given in Table IV show that the ratio of DNA increased to PNA

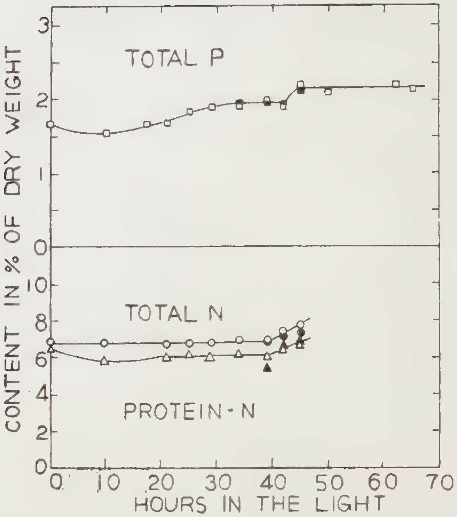

FIG. 7. Percentage (on a dry weight basis) of total nitrogen, protein nitrogen and total phosphorus as found in cells during the course of their transformation.

Open and solid circles and triangles represent, respectively, the values found before and after incubation of cells in the dark.

TABLE IV

Decrease of PNA and Increase of DNA during the Incubation of Light Cells in the Dark

Length of culture under illumination	Increase of DNA-P (in γ per liter of culture) : *a*	Decrease of PNA-P (in γ per lieter of culture): *b*	*a*:*b*
hrs.	$\gamma/l.$	$\gamma/l.$	
34	19.3	64.0	1 : 3.3
39	20.7	88.0	1 : 4.3
42	74.8	141	1 : 1.9
45	30.7	121	1 : 3.8

decreased was about 1 : 2—1 : 4.

In Fig. 8 are given the ratios of PNA-P/(PNA-P+DNA-P), protein-N/total-N, (PNA-P+DNA-P)/total-P, and PNA-P/protein-N. All these ratios, except the value of PNA-P/protein-N at the initial stage of culture, were found to be strikingly constant throughout the whole course of culture. A point of interest is that there exists an almost complete coincidence in the values of PNA-P/(PNA-P+DNA-P) and protein-N/total-N. Whether this coincidence has some significance or not is a question which is left open to further investigations.

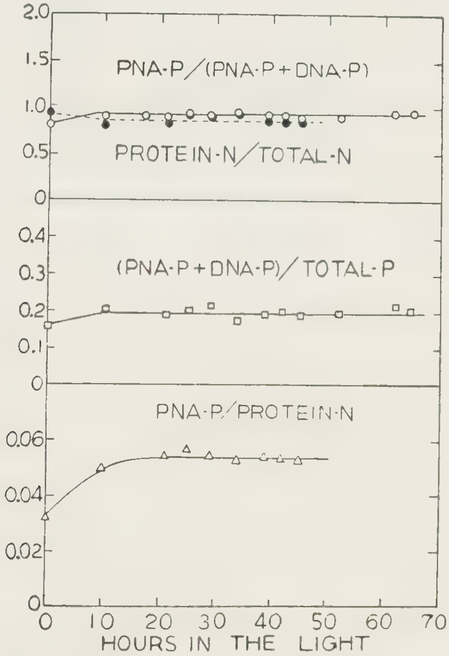

Fig. 8. Ratios of PNA-P/(PNA-P+DNA-P) (open circles); protein-N/total-N (solid circles); (PNA-P+DNA-P)/total-P (squares); and PNA-P/protein-N (triangles) as found in cells during the course of their life cycle.

SUMMARY

1. The life cycle of the green alga *Chlorella* was studied using the technique of "synchronous" culture. Successive stages of the life cycle were conveniently referred to as (i) growing phase, (ii) sporulating phase, and (iii) dividing phase. The growing phase represents the stage of transformation of "dark cells" into "light cells" according to the terminology of Tamiya *et al.* (*1*). The sporulating phase is the stage

of ripening of light cells, and the dividing phase is the stage of formation of a number (n) of dark cells from individual light cells. Average values of n shown by the light cells of increasing grade of ripening were 1.3, 4.7, and 5.1, respectively.

2. The content (in percent of dry weight) of pentose-nucleic acid (PNA) was found to be relatively constant throughout the whole course of cell transformation. In contrast, the percentage of desoxypentose-nucleic acid (DNA) decreased more or less in the growing phase, and increased markedly in the sporulating phase.

3. On incubating the cells in the dark, PNA content decreased, while DNA content increased especially markedly in light cells. The ratio of DNA increased to PNA decreased was found to be about 1 : 2 –1 : 4. Based on these observations it was suggested that in the cell, DNA may be formed, at least partly, from PNA.

4. DNA content per cell of light cells after the incubation in the dark was found to be roughly proportional to the number of dark cells to be produced from individual light cells. This finding is in accord with the prevailing concept that DNA is contained in a constant amount in each nucleus.

5. In respect to total nitrogen and protein nitrogen, cells at various developmental stages showed almost the same content on a dry weight basis. Approximately constant were also the ratios: (PNA-P+DAN-P)/total-P, (PNA-P)/(PNA-P+DNA-P) and PNA-P/protein-N. It was further found that the ratios of PNA-P/(PNA-P+DNA-P) and protein/N/total-N coincided strikingly with each other.

This work was carried out as a part of the studies on the growth of *Chlorella* directed by Prof. Hiroshi Tamiya, to whom the writer is indebted for his guidance. Thanks are also due to Dr. E. Hase and Mr. Y. Morimura for their kind co-operation during the course of this research. This work was aided by grants from the Rockefeller Foundation and the Ministry of Education. To these bodies we extend our grateful thanks.

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"ALLITHIAMINE", A NEWLY FOUND DERIVATIVE OF VITAMIN B_1

III. ON THE ALLICIN HOMOLOGUES IN THE PLANTS OF THE ALLIUM SPECIES

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In the first report (*1*) the authors stated that thiamine lost its characteristic reaction in the garlic extract and changed into allithiamine, and that this phenomenon arose from the chemical reaction of thiamine with allicin as shown by Cavallito *et al.* (*2*). In connection with this phenomenon, we have further found the facts itemized below.

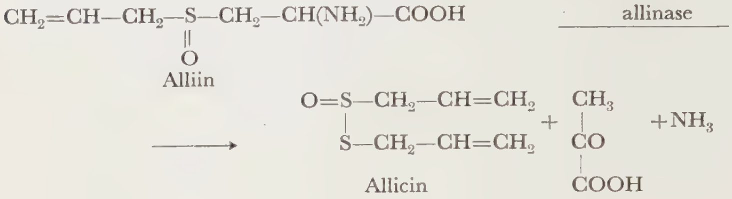
1. Compounds like allithiamine are produced not only in the presence of garlic but of other plants of the *Allium* species, in general, such as stone-leek, onion, leek, and scallion.

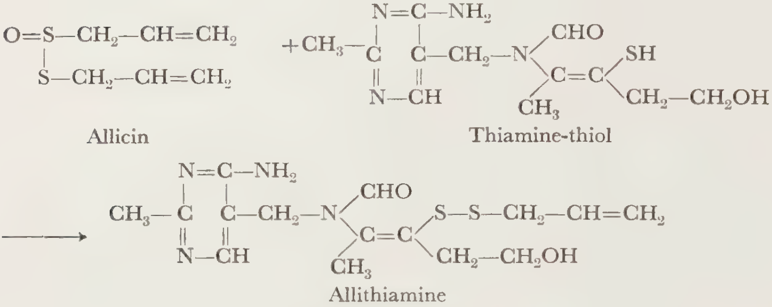
2. The action of the plants of the *Allium* species is brought into effect only when they are ground raw. This fact agrees well with the fact, explained for the first time by Stoll *et al.* (*3*), that allicin is produced from alliin by the action of allinase contained in the plants.

3. The enzyme (allinase), probably responsible for this phenomenon, is common to all the plants of *Allium* species. Thus, when unscathed garlic bulb is boiled prior to grinding, no allicin, which is to act on thiamine, can be found in the extract. However, for instance, when the specimen of the scallion protein obtained from the precipitate formed in the extract of fresh scallion by acidification to pH 4, is added to the extract of the garlic bulb, allicin is immediately produced. Conversely, the protein from garlic has the same effect on the extract of scallion or stone-leek.

4. Pyruvic acid and ammonia have been found not only from the extract of garlic, but from the extracts of any other *Allium* species.

From the above facts it has been concluded that the relation between the ingredients of garlic and thiamine, as shown in the following schema, can probably be applied to all the *Allium* species. We have also inferred that the difference of odor among these plants is caused by the structural difference in the alkyl radicals in allicin.


Alliin
allinase
Allicin


Allicin
Thiamine-thiol
Allithiamine

From the above points of view the compounds corresponding to allicin and alliin in the *Allium* species were investigated by the paper chromatographic method. In the present paper we shall report on a series of studies of allicin.

In the following chapters, for convenience, we shall refer to allicin with the general formula of $R\text{-}\underset{\underset{O}{\|}}{S}\text{-}S\text{-}R$, in which R represents an alkyl radical.

METHODS

I. Paper Chromatography of $R\text{-}\underset{\underset{O}{\|}}{S}\text{-}S\text{-}R$*

Developing Solvent—As the result of various investigations on synthesized $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$*, we came to the conclusion that for the paper chromatography of this compound petroleum ether saturated with 95 per cent methanol was the best suited as the developing solvent.

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* Allicin employed as material was prepared in the following way: Dissolve 0.9 g. of R–S–S–R, diallyl disulfide, for instance, in 6 ml. of acetic acid, and 0.6 g. of H_2O_2(30 per cent), let stand overnight at room temperature, and adjust the solution to neutral pH by the addition of sodium bicarbonate. This neutralized solution was readily used as the material.

Coloration—Coloration was examined by means of sodium nitroprusside and iodine azide. They were prepared as follows:

1) Iodine azide reagent: 0.9 g. of iodine and 1.5 g. of KI were dissolved in 150 ml. of water, to which 2.5 g. of NaN_3 was dissolved.

2) Sodium nitroprusside reagent: 1.5 g. of sodium nitroprusside was dissolved in 5 ml. of 2 *N* H_2SO_4, to which 95 ml. of methanol and 10 ml. of 28 per cent liquid ammonia were added and filtered (*4*).

3) A 5 per cent NaOH solution.

4) A 2 per cent NaCN solution: 2 g. of NaCN was dissolved in 5 ml. of water and 95 ml. of methanol was added to the solution.

By the iodine reagent alone $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$ and R–S–S–R cannot clearly be distinguished. On the other hand, the sodium nitroprusside reagent was quite effective in discrimination between these two. After previous spraying with the nitroprusside reagent, followed by spraying with a 2 per cent NaCN solution, R–S–S–R showed deep reddish purple, while $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$ scarcely gave no color. Conversely, R–S–S–R showed no color with a 5 per cent NaOH solution after previously having been sprayed with nitroprusside reagent, while $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$ gave bright reddish orange or reddish purple color by the same process.

The Rf values of the substances of $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$ type developed by the above-mentioned solvent by the ascending method are shown in the Table I.

II. Differentiation of Alkyl Radicals in $R\text{-}\underset{\underset{O}{\|}}{S}\text{-}S\text{-}R$

As stated above, the allicin homologues showed different Rf values on paper chromatograms. It seems possible, therefore, to differentiate between several alkyl radicals by the aid of Rf values, but as will be stated later (*Result I*), it was found that when the extracts of natural substances were developed on a paper chromatogram, some of them gave Rf values different from those listed above. Consequently, there arose a need to make the differentiation of alkyl radical more precisely. For this purpose, taking advantage of the fact that $R\text{–}\underset{\underset{O}{\|}}{S}\text{–}S\text{–}R$ reacts with cysteine and forms *S*-alkylmercaptocysteine ($R\text{–}S\text{–}S\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH$) (*2*), we developed this on a paper chromatogram following the method adopted by Matsukawa *et al.* (*5*). By the resultant Rf values, alkyl radicals were determined. The procedure was as follows:

A sample was placed on the starting line of a sheet of filter paper (40 cm. square),

TABLE I

Rf Value of $R-\underset{\underset{O}{\|}}{S}-S-R$

Material	Rf Value
$CH_3-\underset{\underset{O}{\|}}{S}-S-CH_3$	0.13
$C_2H_5-\underset{\underset{O}{\|}}{S}-S-C_2H_5$	0.60
$C_3H_5-\underset{\underset{O}{\|}}{S}-S-C_3H_5$	0.80
$C_3H_7-\underset{\underset{O}{\|}}{S}-S-C_3H_7$	0.90

Developing solvent: Petroleum ether saturated with 95 per cent methanol.

and developed with petroleum ether saturated with 95 per cent methanol by the ascending method, as shown in Section I. The guide paper was cut off and sprayed with the sodium nitroprusside and NaOH reagents, by which the positions of the $R-\underset{\underset{O}{\|}}{S}-S-R$ were located. Then a strip of paper corresponding to the desired $R-\underset{\underset{O}{\|}}{S}-S-R$ was cut off from the unsprayed chromatograms. This strip was cut into small pieces and extracted with approximately 50 ml. of absolute ethanol. This extract was concentrated at a low temperature under a reduced pressure. The residual liquid was mixed with 5 ml. of distilled water, about 10 mg. of cysteine-HCl was added to it, and the solution was kept at room temperature. This reactive liquid was submitted to paper chromatography, with the upper layer of a mixture of butanol, ethanol, acetic acid and water 4:1:1:10) as the developing solvent. The Rf values of $R-S-S-CH_2-CH(NH_2)-COOH$ are shown in the Table II.

TABLE II

Rf Values of $R-S-S-CH_2-CH(NH_2)-COOH$

Material	Rf Value
$CH_3-S-S-CH_2-CH(NH_2)-COOH$	0.50
$C_2H_5-S-S-CH_2-CH(NH_2)-COOH$	0.64
$C_3H_5-S-S-CH_2-CH(NH_2)-COOH$	0.67
$C_3H_7-S-S-CH_2-CH(NH_2)-COOH$	0.76

Developing solvent: Butanol—ethanol—acetic acid—water (4:1:1:10). Detection by ninhydrine reagent.

RESULTS

I. Allium sativum L. (*Garlic*)

One hundred g. of fresh bulbs of garlic was ground, and mixed with 200 ml. of absolute ethanol. After habing been left in a refrigerator overnight it was filtered with suction. This filtrate was added to the previous filtrate. After this combined filtrate was concentrated to about 20 ml. at a low temperature under reduced pressure, 40 ml. of ether was added to it. This mixture was shaked vigorously to effect transition of allicin to the ethereal phase. This extraction procedure was repeated twice. The combined extract was concentrated to 10 ml. at a low temperature under reduced pressure and submitted to paper chromatographic analysis.

As a result of chromatographic analysis by the method given in Section I, four spots were detected at Rf 0.13, 0.52, 0.78, and 0.90. The spots at Rf 0.78 and 0.90 were somewhat indistinct in their border. The spot at Rf 0.52 was an unknown one.

Examination of the alkyl radicals was carried out with the same extract by the method mentioned in Section II. Following results were obtained: Only one spot at Rf 0.50, corresponding to CH_3-S-S-CH_2-CH(NH_2)-COOH, was found from the extract of a strip of the paper with Rf 0.13; a distinct spot of C_3H_5-S-S-CH_2-CH(NH_2)-COOH (Rf 0.67) and faint spots of CH_3-S-S-CH_2-CH(NH_2)-COOH (Rf 0.50) and C_3H_7-S-S-CH_2-CH(NH_2)-COOH (Rf. 0.76) from that of Rf 0.78; a spot of C_3H_7-S-S-CH_2-CH(NH_2)-COOH and a faint spot of C_3H_5-S-S-CH_2-CH(NH_2)-COOH (Rf 0.67) from that of Rf 0.9 ; and clear spots of CH_3-S-S-CH_2-CH(NH_2)-COOH (Rf 0.50) and C_3H_5-S-S-CH_2-CH$(NH)_2$-COOH (Rf 0.67) from the unknown spot of Rf 0.52.

Accordingly, it can be concluded that the spot of Rf 0.13 is that of $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}CH_3$, that of Rf 0.78 is of $C_3H_5\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_5$ (allicin) and $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$, that of Rf 0.90 is of $C_3H_5\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$ and $C_3H_7\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$, and that of Rf 0.52 is of $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_5$ only, as shown in Table III.

II. Allium Bakeri Regel (*Scallion*)

Two hundred and fifty g. of scallion was examined by the same method as in the case of garlic. When it was developed with petroleum ether saturated with 95 per cent methanol, three spots appeared at Rf

TABLE III

Paper Chromatogram of the Allicin Homologues in Garlic

Method of Item I. (Paper chromatogram of $R-\underset{\underset{O}{\|}}{S}-S-R$) Rf	Method of Item II. (Paper chromatogram of $R-S-S-CH_2-CH(NH_2)-COOH$)	Allicin homologue
0.12	$CH_3-S-S-CH_2-CH_2(NH_2)-COOH^{*}$	$CH_3-\underset{\underset{O}{\|}}{S}-S-CH_3^{*}$
0.52	$CH_3-S-S-CH_2-CH(NH_2)-COOH^{**}$ $C_3H_5-S-S-CH_2-CH(NH_2)-COOH^{**}$	$CH_3-\underset{\underset{O}{\|}}{S}-S-C_3H_5^{**}$
0.76	$CH_3-S-S-CH_2-CH(NH_2)-COOH^{*}$ $C_3H_5-S-S-CH_2-CH(NH_2)-COOH^{***}$ $C_3H_7-S-S-CH_2-CH(NH_2)-COOH^{*}$	$C_3H_5-\underset{\underset{O}{\|}}{S}-S-C_3H_5^{***}$ $CH_3-\underset{\underset{O}{\|}}{S}-S-C_3H_7^{*}$
0.90	$C_3H_5-S-S-CH_2-CH(NH_2)-COOH^{**}$ $C_3H_7-S-S\ CH_2-CH(NH_2)-COOH^{**}$	$C_3H_5-\underset{\underset{O}{\|}}{S}-S-C_3H_7^{**}$ $C_3H_7-\underset{\underset{O}{\|}}{S}-S-C_3H_7^{*}$

0.12, 0.52 and 0.80. Their alkyl radicals were examined thereafter by the method described in Section II. $CH_3-S-S-CH_2-CH(NH_2)-COOH$ was detected from the extract of the strip of the paper with Rf 0.12, $CH_3-S-S-CH_2-CH(NH_2)-COOH$ and $C_3H_5-S-S-CH_2-CH(NH_2)-COOH$ from that of Rf 0.52, $CH_3-S-S-CH_2-CH(NH_2)-COOH$, $C_3H_5-S-S-CH_2-CH(NH_2)-COOH$, and $C_3H_7-S-S-CH_2-CH(NH_2)-COOH$ from that of Rf 0.80. The results are summarized in Table IV.

III. Allium Grayi Regel

The examination of *Allium* Grayi Regel gave the same results with *Allium* Bakeri Regel.

IV. Allium odorum L.

Two hundred and fifty g. of the bulbs of *Allium odorum* L. was treated and examined in the same way as in the case of garlic. The results are shown in Table V.

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* Very small in quantity. ** Small in quantity. *** Large in quantity.

TABLE IV

Paper Chromatogram of the Allicin Homologues in Allium Bakeri Regel

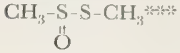
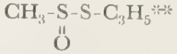
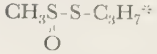
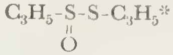
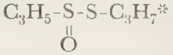
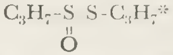
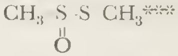
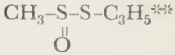
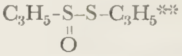
Method of section I. Rf	Method of section II.	Allicin homologues
0.12	$CH_3\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$***	 $CH_3\text{-}\underset{\underset{O}{\Vert}}{S}\text{-}S\text{-}CH_3$***
0.52	$CH_3\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$** $C_3H_5\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$**	 $CH_3\text{-}\underset{\underset{O}{\Vert}}{S}\text{-}S\text{-}C_3H_5$**
0.80	$CH_3\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$* $C_3C_5\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$* $C_3H_7\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$*	 $CH_3\underset{\underset{O}{\Vert}}{S}\text{-}S\text{-}C_3H_7$*  $C_3H_5\text{-}\underset{\underset{O}{\Vert}}{S}\text{-}S\text{-}C_3H_5$*  $C_3H_5\text{-}\underset{\underset{O}{\Vert}}{S}\text{-}S\text{-}C_3H_7$*  $C_3H_7\text{-}\underset{\underset{O}{\Vert}}{S}\ S\text{-}C_3H_7$*

TABLE V

Paper Chromatogram of the Allicin Homologues in Allium odorum L.

Method of Section I. Rf	Method of Section II.	Allicin homologues
0.13	$CH_3\text{–}S\text{–}S\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH$***	 $CH_3\ \underset{\underset{O}{\Vert}}{S}\ \text{-}S\ CH_3$***
0.52	$CH_3\text{–}S\text{–}S\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH$** $C_3H_5\text{–}S\text{–}S\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH$**	 $CH_3\text{–}\underset{\underset{O}{\Vert}}{S}\text{–}S\text{-}C_3H_5$**
0.80	$C_3H_5\text{–}S\text{–}S\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH$**	 $C_3H_5\text{-}\underset{\underset{O}{\Vert}}{S}\text{–}S\text{-}C_3H_5$**

V. Allium victorialis L.

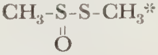
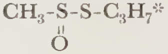
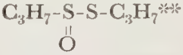
The examination of *Allium victorialis* L. yielded the same result as with *Allium odorum* L.

VI. Allium fistulosum L.

The results of treating 500 g. of the *Allium fistulosum* L. in the same manner as with garlic are shown in Table VI. The C_3H_5-radical could not be detected in this plant.

TABLE VI

Paper Chromatogram of the Alllcin Homologues in Allium fistulosum L.

Method of section I. Rf	Method of section II.	Allicin homologues
0.12	CH_3-S-S-CH_2-CH(NH_2)-COOH*	 $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}CH_3$*
0.72	CH_3-S-S-CH_2-CH(NH_2)-COOH* C_3H_7-S-S-CH_2-CH(NH_2)-COOH*	 $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$*
0.90	C_3H_7-S-S-CH_2-CH(NH_2)-COOH**	 $C_3H_7\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$**

VII. Allium cepa L. (Onion)

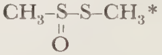
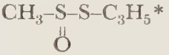
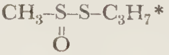
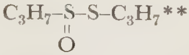
With onion the same results as with *Allium fistulosum* L. were obtained.

VIII. Allium fistulosum L. var. caespitosum Makino

Two hundred and fifty g. of *Allium fistulosum* L. *var. caespitosum* Makino was examined. The results are summarized in Table VII.

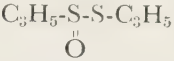
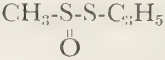
TABLE VII

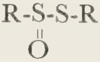
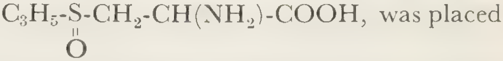
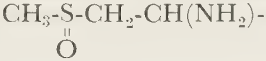
Paper Chromatogram of the Allicin Homologues in the Allium fistulosum L. var. caespitosum Makino

Method of Section I. Rf	Method of Section II.	Allicin homologues
0.13	CH_3-S-S-CH_2-CH(NH_2)-COOH*	 $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}CH_3$*
0.52	CH_3-S-S-CH_2-CH(NH_2)-COOH* C_3H_5-S-S-CH_2-CH(NH_2)-COOH*	 $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_5$*
0.72	CH_3-S-S-CH_2-CH(NH_2)-COOH* C_3H_7-S-S-CH_2-CH(NH_2)-COOH*	 $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$*
0.90	C_3H_7-S-S-CH_2-CH(NH_2)-COOH**	 $C_3H_7\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_7$**

DISCUSSION

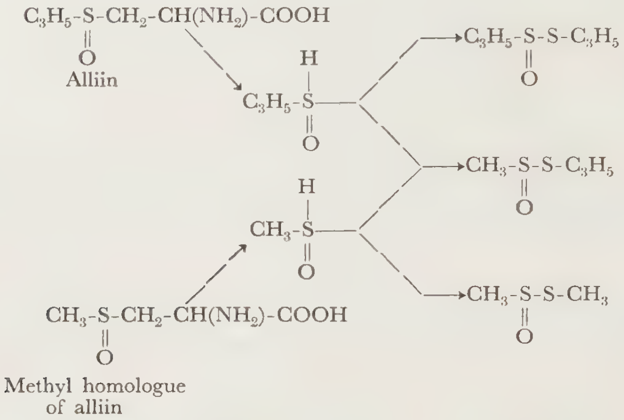
The effective ingredients of garlic have been described by Cavallito (*2*) and Stoll (*3*), who showed that garlic contains alliin, which, by the action of allinase, changes into allicin and emits the peculiar odor, but of the effective ingredients of the rest of the *Allium* species plants almost nothing has been found so far as we are aware, except of the report of Matsukawa *et al.* (*5*), our coworkers. Such being the case, it was more than we had expected that we found that garlic and thiamine combined chemically to produce allithiamine, and that this phenomenon holds not only for garlic but for all the rest of the *Allium* species. We were fortunate enough to find an excellent approach to the analysis of the componental parts of the *Allium* species plants.

As a result of these investigations, it was found that the *Allium* species contained not only allicin, *i.e.*,  $C_3H_5\text{-}\underset{\substack{\| \\ O}}{S}\text{-}S\text{-}C_3H_5$ discovered by Cavallito, but allicin homologues, alkyl radical of which were replaced by CH_3- or C_3H_7-. Beside these, there is an instance like  $CH_3\text{-}\underset{\substack{\| \\ O}}{S}\text{-}S\text{-}C_3H_5$ whose two alkyl radicals are different.

In order to ascertain the existence of  $R\text{-}\underset{\substack{\| \\ O}}{S}\text{-}S\text{-}R'$ and the process of its generation, the following experiments were carried out: In the first case, synthesized alliin, *i.e.*  $C_3H_5\text{-}\underset{\substack{\| \\ O}}{S}\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$, was placed under the action of allinase taken from garlic; in the second case, the methyl homologue of alliin, *i.e.*  $CH_3\text{-}\underset{\substack{\| \\ O}}{S}\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$, was placed under the action of allinase; in the third case, the mixture of these two, previously mixed, was placed under the action of allinase. Under these three conditions, the allicin homologues formed in test tubes were examined by the same method as employed previously. It was thereby found that only one spot at Rf 0.80 was observed from the reaction product of alliin alone and allinase, and one spot of Rf 0.13 from the methyl homologue of alliin. In the third case, where the mixture of alliin and its methyl homologue underwent the action of allinase, three spots were observed at Rf 0.13, 0.52, and 0.80.

In the next step, by the same method examination as described in Section II for natural substances, cysteine was reacted with extracts of these spots, and the resultant $R\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$ was submitted to paper chromatography. As was expected, a spot of $CH_3\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$ was produced from that of Rf 0.13 in each case, and a spot of $C_3H_5\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$ from Rf 0.80, but two spots of $CH_3\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$ and $C_3H_5\text{-}S\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$ from Rf 0.52. These relationships are the same as the result of examination of natural substances. Consequently we may assume that the allicin homologue at Rf 0.52 is that of $CH_3\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_5$. It was also proved that the same relation holds for a mixture of methyl and propyl homologues of alliin reacted with allinase.

It may be inferred that when allicin is produced from alkyl sulfenic acid (*3*) different alkyl radicals undergo mutual exchange an allicin homolugue like $R\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}R'$ is produced as shown below.



It has always been the habit to look in the plants of *Allium* species for diallyl disulfide which was first reported by Semmler (*6*), but the compound was never detected in any of the present series of experiments. However, diallyl disulfide was always found in the steam-distilled liquid of garlic. Consequently, we might surmise that a greater part of allicin changes into diallyl disulfide through steam-distillation.

SUMMARY

Allicin in the extract of *Allium* species plants was examined by the paper chromatographic method, and the following results were obtained :

1. Allicin ($C_3H_5\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}C_3H_5$) or its homologues ($R\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}R$ or $R\text{-}\underset{\overset{\|}{O}}{S}\text{-}S\text{-}$

R′) is generally present in the plants of *Allium* species.

2. As the alkyl radicals of allicin homologues, CH_3-, C_3H_5-, and C_3H_7- are found, but not C_2H_5 in the natural products. These results coincide well of the presumptions of Matsukawa *et al.* (*5*).

The authors express their hearty gratitude to Dr. U. Miura, Kyoto University, for encouragement, to Dr. T. Matsukawa and Dr. S. Yurugi, Research Laboratory, Takeda Pharmaceutical Industries, Ltd., for offer of help in various ways. The experiments were aided by a grant from the Ministry of Education.

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(*3*) Stoll, A., and Seebeck, E., *Helv. Chim. Acta*, **31**, 189 (1948)
(*4*) Toennies, G., and Kolb, J. J., *Anal. Chem.*, **23**, 823 (1951)
(*5*) Matsukawa, T., Yurugi, S., and Matsuoka, T., *Science*, **118**, 325 (1953)
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STUDIES ON TAKA-AMYLASE A

IV. ON THE AMINO-TERMINAL AMINO ACID OF TAKA-AMYLASE A

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A number of amylases have been isloated in pure crystalline state and three of them, pig pancreas amylase, taka-amylase A (TAA), and human salivary amylase, have been reported on their amino acid composition (*1*, *2*, *3*). Nevertheless, no publications are so far available for the elucidation of the amylase activity in terms of its chemical structure.

In the authors' laboratory, a simple method for the preparation of crystalline TAA has been achieved (*4*), by which a sufficient quantity of this amylase was obtained for extensive studies on its chemical structure.

The first step onto the study of the chemical structure of proteins is to characterize and determine their amino- and carboxyl-terminal groups. The present paper deals with the determination of the *N*-terminal amino acid of TAA and the estimation of its minimum molecular weight.

MATERIALS

Taka-amylase A—Crystalline TAA prepared from " Takadiastase Sankyo " was recrystallized three times from aqueous acetone as described in the previous paper (*4*).

Dinitrophenyl-taka-amylase A (*DNP-TAA*)—To about 400 mg. of TAA dissolved in 5 ml. of water, 0.5 g. of solid $NaHCO_3$ and 0.5 ml. of dinitrofluorobenzene in 10 ml. of ethanol were added and kept shaking in the dark. After 2 hours the precipitated DNP-protein was centrifuged, and washed successively with water, ethanol and ether, and dried in vacuum. DNP-TAA thus obtained is an amorphous, yellow powder, insoluble in water and organic solvents. The amount of original protein in the DNP-derivative was estimated by determing the amide content of the DNP-protein.

Three to five mg. of DNP-TAA were heated with 2 ml. of 2 *N* HCl in a sealed tube for 3 hours at 105–110°. The hydrolysate was neutralized with NaOH to pH 3–5 in the ice cold. This solution was transferred to a micro Kjeldahl apparatus, an excess of $M/5$ borate buffer (pH 9.0) was added, ammonia liberated was distilled by the usual Kjeldahl method and absorbed in 5 ml. of $N/100$ H_2SO_4. The content of ammonia in this solution was determined by photometric ninhydrin method according to Moore and Stein (*5*).*

Sample No.	DNP-TAA Amount	Liberated NH_3	Amide NH_3
I	3.306 *mg.*	37.2 γ	1.13 %
I	3.660	40.6	1.11
II	5.575	59.2	1.06
II	4.595	48.0	1.02

Solvent Systems Employed—DNP-amino acid was identified by the Sanger's method (*7*).

The solvent systems employed are as follows.

Solvent system A: Chloroform-water
Solvent system B: 1% butanol, chloroform-water
Solvent system C: 70% methyl ethyl ketone, ether-water

EXPERIMENTAL

Identification of N-Terminal Amino Acid—Fifty mg. of DNP-TAA were hydrolyzed with 2 ml. of 6 N HCl in a sealed tube by heating to 105–110° for 15 hours. The hydrolysate was diluted with 10 ml. of water and extracted three times with 10, 7 and 5 ml. of ether, respectively. The combined extracts were washed with a small volume of water, and ether was expelled. The residue was fractionated on a silica gel column using the solvent system A, whereby two yellow bands were developed.

The faster moving band consisted of dinitroaniline and dinitrophenol. The slower band ($R_c=0.18$)** was again chromatographed on a silica gel column using the solvent system B. The band ($R_c=0.48$) by this solvent system was in accord with that of DNP-alanine. The band was cut, eluted with ethyl acetate and the amino acid was regenerated by Lowther's method (*8*). The free amino acid produced was identified as alanine by usual paper chromatography.

Identification of ε-DNP-lysine—The aqueous phase of the hydrolysate of DNP-TAA, after being extracted with ether, was diluted to 25 ml. An aliquot of the solution was dried in vacuo and chromatographed on a silica gel column using the solvent system C. A band ($R_c = 0.22$) was in accordance with that of ε-DNP-lysine. The absence of mono-DNP-arginine and di-DNP-histidine in the aqueous phase was confirmed by the method of Desnuelle *et al.* (*9*).

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* For the microdetermination of ammonia the authors are indebted to Mr. Y. Okada and H. Hanafusa (*6*).

** R_c value expresses the ratio of moving distance of DNP-amino acid to that of the front of developing solvent, as Rf value in the paper chromatography.

Recovery Factor of DNP-alanine during Acid Hydrolysis—DNP-amino acids are partly decomposed during acid hydrolysis of DNP-protein, and DNP-alanine has been reported to be one of the most labile DNP-amino acid. (*10*, *11*) The recovery factor of DNP-alanine was determined as follows.

One hundred to three hundred γ of DNP-alanine, DNP-protein being added or not added, were heated with 6 N HCl in a sealed tube. After cooling DNP-alanine was extracted with ether and chromatographed on a silica gel column. The band of DNP-alanine was extracted with ethyl acetate, dissolved in 1 per cent $NaHCO_3$ and estimated by a photoelectric colorimeter. Table I shows recovery factors in the experiments of various time of heating. Correction factor for any time of acid hydrolysis can be obtained from the curve shown in Fig. 1.

TABLE I

Recovery of DNP-alanine during Acid Hydrolysis

DNP-alanine	DNP-TAA	Time of hydrolysis	DNP-alanine recovered	Recovery Factor
γ	*mg.*	*hrs.*	γ	%
200	—	2	190	95
200	—	4	178	89
200	—	6	164	82
200	—	8	154	77
141	50	6	114	81
286	20	8	246	86
141	30	15	81	57

Quantitative Determination of N-Terminal DNP-alanine and the Minimum Molecular Weight of TAA—Thirty to fifty mg. of DNP-TAA were hydrolyzed with 6 N HCl, DNP-alanine produced was separated chromatographically and determined colorimetrically. For the qunatitative

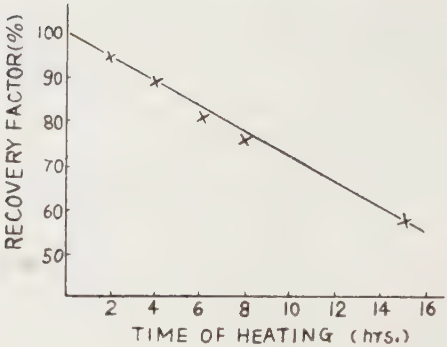

Fig. 1. Recovery factor of DNP-alanine during acid hydrolysis.

determination of *N*-terminal amino acid of DNP-TAA 6 to 8 hours hydrolysis at 105–110° is the most favorable, because *N*-terminal DNP-alanine is liberated easily by the acid hydrolysis and decomposed by prolonged heating. The results of experiments are summarized in Table II. The molecular weight was calculated on the assumption that TAA bears one *N*-terminal alanine.

DNP-TAA used in expt. No. 8 and No. 9 was prepared from TAA denatured by incubation with hydrochloric acid (pH 1.5) at 37° for 2 hours. Amounts of alanine given in the 5th column of Table II are values corrected for destruction during the acid hydrolysis.

Quantitative Determination of ε-DNP-lysine—In the expt. No. 7 and No. 9 of Table II. ε-DNP-lysine besides DNP-alanine was separated and colorimetrically determined. In these two experiments ε-DNP-lysine estimated were 12.7 and 13.5×10^{-6} moles, corresponding to 18.7 and 18.5 moles, respectively, per mole TAA.

ε-DNP-lysine is very stable against heating with hydrochloric acid. When 3.13 mg. of ε-DNP-lysine were heated with 2 ml. of 6 N HCl, in the presence of 30 mg. of DNP-TAA, in a sealed tube at 105–110° for 15 hours, the recovery was as high as 90–95 per cent. If the correction factor 100/90 for 15 hours acid hydrolysis is applied, 18.5 and 18.7 moles correspond to 20.6 and 20.8 moles. These values are in good agreement with the result of amino acid analysis of TAA reported previously (*2*).

SUMMARY

The dinitrofluorobenzene method has been applied to the iden-

Table II

N-Terminal Alanine and Molecular Weight of Taka-amylase A

No. of experiment	DNP-TAA	TAA	Time of hydrolysis	DNP-alanine	Molecular weight
	mg.	*mg.*	*hrs.*	10^{-7} *M*	
1	106.5	85.3	6	17.0	50,000
2	39.5	31.8	6	6.9	46,000
3	37.8	30.4	8	6.0	51,000
4	38.0	26.6	6	4.6	58,000
5	38.8	27.2	8	5.1	53,000
6	50.0	35.8	6	6.6	54,000
7	50.0	35.8	15	6.7	53,000
8	50.0	38.5	6	7.2	54,000
9	50.0	38.5	15	7.2	54,000
				average	52,500

tification and quantitative determination of amino-terminal amino acid of taka-amylase A. Alanine was found to be a single amino-terminal amino acid. From quantitative studies, the minimum molecular weight of taka-amylase A was estimated as 52,500.

The authors wish to express their gratitudes to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo" and to Mr. Y. Okada for his technical assistance.

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LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 42, No. 5, 1955

A NEW CRYSTALLINE FORM OF CHYMOTRYPSIN

Sirs:

In the previous papers (*1*, *2*, *3*, *4*), it was reported by the authors that the slow activation of α-chymotrypsinogen by trypsin, was a highly complex process accompanied with the formation of a number of intermediate proteins or liberated peptides. Crystalline α-chymotrypsin, which can be isolated after slow activation of the zymogen for 48 hours in approximately 50 per cent yield (*5*), contains two amino-(N-) terminal groups, *i.e.* alanine and isoleucine (*2*, *6*) and the slow activation involves the opening of cyclic polypeptide chains of the zymogen, yielding alanine at first and then isoleucine as *N*-terminal groups of α-chymotrypsin (*2*).

An attempt to prepare crystalline α-chymotrypsin by the slow activation led the author to isolate a new crystalline protein, which was identical with none of the known crystalline chymotrypsins, as far as crystalline form concerned, and was supposed to be a new crystalline chymotrypsin.

Crystalline bovine α-chymotrypsinogen filter cake (8.2 g.) was suspended in 24.6 ml. of water, and dissolved by adding several drops of 5 *N* sulfuric acid, and then 8.2 ml. of *M*/2 phosphate buffer (pH 7.6) and a quantity of 5 *N* sodium hydroxide equivalent to the acid were added. The mixture was cooled to 5° in a cellophane bag, and dialyzed against 40 ml. of *M*/8 phosphate buffer (pH 7.6). Then the mixture was mixed with 0.82 ml. of trypsin solution containing 0.43 mg. of trypsin protein, and activated for 48 hours at 5° to the maximum activity. Dialyzing buffer was replaced by a new buffer every ten hours and each of the buffer was cooled to 5° before use.

The activated mixture was acidified to pH 4.0 by 4 *N* sulfuric acid, added by 18 g. of solid ammonium sulfate, and filtered with suction. Further experiment was made according to the method of Kunitz and Northrop (*5*) to obtain crystalline α-chymotrypsin. The amorphous filter cake (7.8 g.) was dissolved in 5.9 ml. of *N*/100 sulfuric acid, and allowed to stand at 20° for a night. Crystallization takes place slowly, but the crystalline form (Fig. 1) differs from that of any known crystalline chymotrypsin.

The cystalline filter cake (2.2 g.) was dissolved in 3.3 ml. of *N*/100 sulfuric acid: about 1 volume of saturated ammonium sulfate was added cautiously until crystallization commences. The solution was allowed to stand at 20°, and practically complete crystallization took place within about one hour.

The crystalline filter cake (1.8 g.), which was recrystallized twice and has yet the

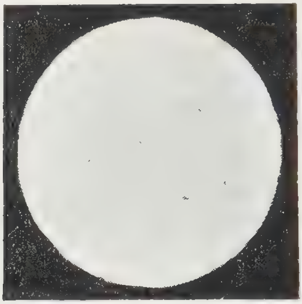

FIG. 1. A new crystalline chymotrypsin (×420).

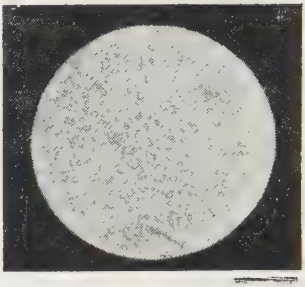

FIG. 2. *α*-Chymotrypsin (Rhombohedron).

same crystalline form as before, was stored in refrigerator for two days and then operated according to the method for recrystallization of *α*-chymotrypsin (*5*). The resulted crystals has come to get the same crystalline form as that of *α*-chymotrypsin (Fig. 2). Like *α*-chymotrypsin, it could be recrystallized in rhombohedrons at pH 4.0–5.0, but in prisms at pH 5.2–6.0.

According to the studies of the rapid activation of α-chymotrypsinogen, mediated by relatively high concentration of trypsin, Jacobsen (*7*) postulated that an initial tryptic hydrolysis yields an unstable intermediate, π-chymotrypsin, which was transformed by further tryptic hydrolysis to δ-chymotrypsin or by chymotryptic hydrolysis to α-chymotrypsin. A further study of the rapid activation by Bettelheim and Neurath (*8*), established experimental proof for its details. They suggested that chymotryptic hydrolysis leads π-chymotrypsin to δ-chymotrypsin, which is degraded autocatalytically to another chymotrypsin and this new intermediate gives rise gradually to α-chymotrypsin.

In the present experiment, a new crystalline chymotrypsin was obtained by the slow activation and it has a different crystalline form from that of any chymotrypsin previously crystallized by Northrop and Kunitz (*5*). It was also found that it can be converted almost quantitatively into α-chymotrypsin during repeated recrystallization and relatively short storage. Therefore, it does not correspond to π-and δ-chymotrypsin. The possibility exists, however, that it might be correspond to another chymotrypsin described by Bettelheim and Neurath (*8*).

In the present study, homogeneity of the new crystalline chymotrypsin was also examined by paper chromatography (developing agent, *n*-butanol :acetic acid :water=4 :1 :1) and except one slow moving spot (corresponds to protein) a rapid moving spot (Rf=0.45) was detected at every crystallization stage, but not after the conversion to α-chymotrypsin. Acid hydrolysate of the spot was proved to consist of two or three different amino acids. As a possible explanation, it may be postulated that in the slow activation the zymogen is converted to the new chymotrypsin as an intermediate and the chymotryptic or spontaneous hydrolysis of the new chymotrypsin gives rise quantitatively to α-chymotrypsin liberating a neutral small peptide.

The elimination of the initially liberated peptide (perhaps basic peptide) by dialysis would depress the rate of the splitting of a bond involving in the conversion of the intermediate to α-chymotrypsin, or inhibit the transpeptidation which was previously postulated by one of the authors *(1)*.

The authors wish to thank Proffessor Shiro Akabori for his guidance throughout this work.

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DETERMINATION OF HYDROGENASE BY THE HYDROGEN EVOLUTION FROM REDUCED METHYLVIOLOGEN

Sirs:

The methods used for the determination of hydrogenase activity in the past are based on either one of the following two principles. (a) Measurement of exchange of deuterium between water and hydrogen or of ortho-, para-conversion of gaseous hydrogen, (b) measurement of reduction of hydrogen acceptors by gaseous hydrogen.

This paper deals with the liberation of gaseous hydrogen from reduced dyes by the reverse reaction of hydrogenase, a phenomenon discovered by Ishimoto (*1*) in our laboratory. In the system consisting of sodium hydrosulphite, methylviologen and hydrogenase, we observed the liberation of gaseous hydrogen, which was measured either manometrically (Fig. 1), or by colorimety of the reduced methylviologen.

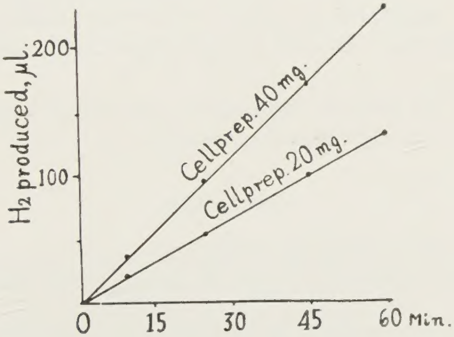

FIG. 1. Hydrogen evolution from reduced methylviologen by the action of hydrogenase. The reaction mixture (3 ml.) in the Warburg flask consisted of 1 ml. of 0.67 *M* phosphate buffer, pH 7.0, 1 ml. of 0.0025 *M* methylviologen chloride and 1 ml. of suspension of acetone dried preparation of proteus vulgaris. Gas phase, 100 percent nitrogen; temperature, 30°. Alkaline pyrogallol solution was put in the center well. At the beginning of the reaction, 36.5 mg. of solid sodium hydrosulphite was added from the side arm.

When any one of the above components of the reaction mixture was omitted or a boiled enzyme preparation was used, the reaction did not take place at all.

Recently, Fisher, Krasna and Rittenberg (*2*) showed that hydrosulphite markedly activated hydrogenase activity as measured by the deuterium exchange reaction. Under our experimental conditions, the hydrosulphite activates the enzyme and reduces methylviologen, which becomes direct substrate for the enzyme.

The reaction proceeds linearly at a rate proportional to the amount of the enzyme as well as methylviologen up to the saturation point. At the saturation point, other variables may be measured most effectively. Methylviologen was successfully replaced by benzylviologen, but not by acriflavin, phenosafranin, neutral red, diphosphopyridine nucleotide and flavin adenine dinucleotide. Other reducing tested so far, namely, stannous chloride, hypophosphorous acid, cysteine and thioglycolic acid, could not replace hydrosulphite. But formic acid—formic dehydrogenase system was found to replace it to some extent. Packer and Vishniac (*3*) reported that they observed hydrogen gas evolution by the action of hydrogenase on reduced diphosphopyridine nucleotide, which was generated by glucose-6-phosphate-glucose-6-phosphate dehydrogenase system. Details of their experiments have not yet been published.

The work of the present method is now being continued to compare deuterium exchange reaction method.

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